expected, the first effect is negligible compared to the second, and it is found experimentally that the polarization of the fluorescence from a turbid solution is always lower than one of the same characteristics from a transparent medium. Fortunately, the Tyndall effect shown by dilute protein solutions seems to have no detectable effect on the polarization. It is a simple matter to detect in any given case whether depolarization by forward scattering takes place. With the mirror M_2 in position, and the exciting beam traversing the middle of the cell, the average path of the fluorescent light inside the solution is about twice the path in the absence of the mirror. If the polarization of the fluorescence is the same with and without the mirror any depolarization by turbidity can be excluded.

SUMMARY

1. A simple addition law for the polarizations of several fluorescent components in solution has been derived.

2. It is shown that a system of components which differ in molecular size or in their lifetime of the excited state, but which follow independently Perrin's law of depolarization give in the plot of $1/p$ against T/η a curve concave with respect to the latter axis. (p = polarization; T = absolute temperature; η = viscosity of the solvent.)

3. An extension of Perrin's theory of depolarization to the case of ellipsoidal molecules carrying randomly oriented oscillators of absorption and emission is described. In such case the polarization of the fluorescence is an explicit function of the principal relaxation times of the rotation of the ellipsoid, the lifetime of the excited state and the limiting polarization.

4. A qualitative treatment of the depolarization by intramolecular rotations is given.

5. The experimental determination of the polarization is described and some causes of error discussed in detail.

The author wishes to express his thanks to Prof. F. Perrin and Dr H. E. Daniels for their suggestions.

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Polarization of the Fluorescence of Macromolecules

2. FLUORESCENT CONJUGATES OF OVALBUMIN AND BOVINE SERUM ALBUMIN

By G. WEBER*
Biochemical Laboratory, University of Cambridge

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In the preceding paper (Weber, 1952) the theory of the polarization of the fluorescence given by macromolecules in solution carrying randomly oriented linear oscillators has been examined. If the relaxation time of the rotation of proteins is to be obtained by this method, the fluorescent oscillators must be rigidly bound to the molecule. Proteins as such are wholly non-fluorescent, but the radiation emitted by a stable complex of the protein with a small fluorescent molecule should have the same optical characteristics as a fluorescence of the protein molecules themselves.

* Beit Memorial Fellow.

Such a stable fluorescent complex can in theory be obtained in several ways: (i) by coupling of a small molecule through a covalent bond and subsequent elimination of the non-coupled fluorescent molecules by the ordinary methods of protein purification; (ii) by adsorption equilibrium of fluorescent molecules on the protein. It will be necessary in the latter case to determine the fraction bound to the protein by an independent method or to consider only the polarization values obtained under conditions such that the whole of the dye is bound to the protein. This last condition requires an affinity of the protein for the fluorescent molecule which is

lacking in most proteins with the conspicuous exception of serum albumin. (iii) By use of a low molecular weight substance which becomes fluorescent on adsorption to the protein. This method is free from the objections of the preceding one, and also from the objection that the protein is being chemically modified as is the case when a fluorescent molecule is attached by covalent bond. We shall show in a later paper that a family of substances exists which, although non-fluorescent in ordinary water solution, become strongly fluorescent when adsorbed.

The present paper refers to the first possibility, namely observations on stable fluorescent conjugates.

It has already been shown (Weber, 1952) that the radiation emitted by a collection of small fluorescent units attached with random orientation to macromolecules which are flat ellipsoids or prolate ellipsoids of small elongation follows a law of depolarization analogous to that first proposed by Perrin (1926) for spherical molecules, namely

$$\frac{1}{p} \mp \frac{1}{3} \simeq \left(\frac{1}{p_0} \mp \frac{1}{3}\right)\left(1 + \frac{3\tau_0}{\rho_h}\right). \qquad (1)$$

Here p is the degree of polarization of light emitted at right angles to the direction of the excitation, τ_0 the lifetime of the excited state of the fluorescence, and ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule. The negative signs correspond to excitation with polarized light vibrating normally to the directions of excitation and observation, the positive signs to excitation by natural light; p_0 is an empirical constant dependent often on the exciting wavelength (but not on τ_0) and in the case of macromolecules (Weber, 1952) perhaps dependent on the existence of intramolecular rotations having a relaxation time much shorter than ρ_h and largely independent of the viscosity of the solvent. This paper describes the preparation and properties of conjugates obtained by reaction of 1-dimethylaminonaphthalene-5-sulphonyl chloride with ovalbumin and bovine serum albumin. Measurements of the polarization of the radiation at different temperatures allow the validity of Eqn. 1 to be tested and the values of ρ_h in the two conjugates and in the same conjugate under different conditions to be compared.

In order to obtain reliable results the following requirements must be met:

(1) The coupling should result in a minimum of chemical change of the protein molecule.

(2) As far as possible only one type of bond should be formed between the protein and the coupled molecule. The formation of comparable amounts of different bonds may result in the protein carrying oscillators with widely different lifetimes of the excited state.

(3) The conjugate must have a fluorescent efficiency comparable to that of the non-conjugated fluorescent substance. If the excess of the latter, which has failed to couple, has a much stronger fluorescence than the conjugate, a very exhaustive purification of the protein will be necessary in order to obtain reproducible results.

(4) A conjugating bond more stable than those responsible for the macromolecular structure of the protein may be required. Such a bond must be stable over the whole range of pH at temperatures below 100°, if the effect of temperature up to that capable of inducing denaturation is to be explored.

(5) It is desirable that the fluorescence should persist at high and low pH values. This results in a serious restriction, since in many substances the fluorescence disappears in acid or alkaline solution. If additional corrections are to be avoided in the comparisons of relaxation times obtained at different pH values, it is necessary that the quenching by acid or alkali should not affect the lifetime of the excited state of the fluorescence. No general rule can be given on this point. For example, the quenching of the fluorescence of riboflavin by acid is accompanied by decrease in the lifetime of the excited state, though not that of eosin (Weber, 1948).

The evidence to be presented in this paper shows that these conditions are met by the conjugates of serum and egg albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride. The coupling does not induce any observable denaturation of ovalbumin, as judged by the solubility at the isoelectric point; experiments with fumarase and ribonuclease indicate that the enzyme activity of conjugates containing 1–3 mol. of naphthalene per mol. of protein is comparable to that of the untreated protein. Although the formation of only one type of linkage cannot at present be demonstrated, this is rendered very likely by the large difference in affinity of the sulphonyl chloride for the —OH and =NH as compared to —NH_2 groups. The stability of the —SO_2NH— bond is well known. In no experiment was there any evidence found of the breaking down of the conjugate as shown by the appearance of the free naphthalenesulphonic acid. The fluorescence of the conjugates was not conspicuously affected by changes in the pH between 1·6 and 14.

From Eqn. 1 it is easily shown that if $p_{\text{min.}}$ denotes the smallest polarization that can be measured with standard error ϵ the range of values of the ratio ρ_h/τ_0 that can be measured with that precision is given by

$$\frac{3+p_0}{3\epsilon} > \frac{\rho_h}{\tau_0} > \frac{3+p_0}{\dfrac{p_0}{p_{\text{min.}}} - 1}.$$

In the sulphonamido conjugates studied p_0 (excitation with natural light) = 0·25; $p_{min.} = 0·1$; and $\epsilon = 0·02$. Therefore,

$$55 > \frac{\rho_h}{\tau_0} > 2.$$

If τ_0 is of the order of 10^{-8} sec. we may expect measurable variations in the polarization for molecules having relaxation times of 10^{-7} sec. order of magnitude. In this range fall the harmonic means of the relaxation times of the rotation of most of the globular proteins studied by the dielectric dispersion method (Oncley, 1942).

EXPERIMENTAL

The technique and apparatus for the polarization determinations have already been described (Weber, 1952). In the experiments described here only excitation with natural light was employed. The measurements of fluorescent intensity were made with a modified Pulfrich photometer. For both the polarizations and intensity observations the exciting light (Hg arc) was filtered through a 5850 Corning glass filter, while the fluorescence was observed through a 335 Corning glass filter. The excitation was due to the Hg lines at 366, 404 and 436 mμ. The values of p_0 obtained are therefore composite values resulting from polychromatic excitation. For reasons indicated below, the viscosities of the solutions were assumed to be those of the pure solvent. The values of Bingham and White (water) and Bingham and Jackson (sucrose solutions), as given by Bingham (1922) were used in the calculations. The absorption spectra were measured with a Beckman quartz spectrophotometer.

Materials

Ovalbumin. Preparations *A* and *C* were three and five times recrystallized ovalbumin prepared by the author (*A*) and by Dr K. Bailey (*C*). Preparation *B*, obtained from Dr A. C. Chibnall, was originally prepared by Prof. R. K. Cannan. It had been stored at room temperature as dry crystals for more than 10 years. About 10% of it was insoluble at pH 4·75, 0·1 ionic strength. Only the fraction soluble at the isoelectric point was used in the preparation of the conjugate.

Serum albumin. Crystalline bovine serum albumin (Armour Laboratories Batch nos. 10,522 and 14,656) was used throughout.

Polylysine. This was prepared by Dr C. S. Hannan (in the Press) by a modification of the method of Katchalski, Grosfeld & Frankel (1948).

Sucrose was a commercial product, the reducing power of which was equivalent to 7 parts of glucose in 100,000 (Benedict). The specific rotation was $[\alpha]_D^{20°} = 66·7°$ in water (24% w/v).

1-Dimethylaminonaphthalene-5-sulphonyl chloride (*V*). 1-Dimethylaminonaphthalene-5-sulphonic acid (I) is easily prepared by methylation of the technical 1-aminonaphthalene-5-sulphonic acid (Fussgänger, 1902). The yield is about 80% of recrystallized acid. For the preparation of the chloride 2·5 g. of the sulphonic acid are ground in a mortar with 3·5 g. of PCl_5 and the resulting yellowish melt poured on water. The insoluble chloride is exhaustively washed with water, filtered and dried over $CaCl_2$. The crude dry powder is extracted successively with acetone and with M-Na_2CO_3. The alkaline extract contains usually 30–50% of unconverted acid. The acetone extract is diluted with 6 vol. of water whereupon the chloride separates as yellow or orange crystals. Yield: 25–40%, m.p. 69°. (Found: S, 12·0; Cl, 13·6. $C_{12}H_{12}O_2NSCl$ requires S, 11·9; Cl, 13·4%.)

The sulphonyl chloride is little affected by water and can be kept for months over $CaCl_2$ without apparent change. It is soluble in acetone, pyridine, benzene and dioxan, insoluble in water. It reacts readily with ammonia and aliphatic amines, much less readily with aniline and very slowly with water or ethanol.

1-Dimethylaminonaphthalene-5-sulphonamide (*II*). 1-Dimethylaminonaphthalene-5-sulphonyl chloride (240 mg.) was dissolved in 2 ml. acetone and 1 ml. of strong ammonia was added. The sulphonamide began to crystallize immediately. Yield: 200 mg. The sulphonamide was recrystallized from ethanol in long colourless needles which showed no loss of weight after 24 hr. at 115°: m.p. 215° (decomp.). (Found: C, 57·5; H, 5·1; N, 11·2; S, 12·7. $C_{12}H_{14}O_2N_2S$ requires C, 57·5; H, 5·5; N, 11·3; S, 12·8%.)

1-Dimethylaminonaphthalene-5-(N-phenyl)-sulphonamide (*III*). Freshly distilled aniline (0·1 ml.) and 269 mg. of sulphonyl chloride dissolved in 1·5 ml. of pyridine were heated in the water bath until no further change in colour was noticed (1–2 hr.). On addition of water a precipitate separated. This was dissolved by warming in 70% ethanol-water and, on cooling, the anilide crystallized in thin green needles. The crystals showed no loss of weight after 24 hr. at 115°, m.p. 141–142°. (Found: C, 66·3; H, 5·8; N, 8·7; S, 9·8. $C_{18}H_{18}O_2N_2S$ requires C, 66·5; H, 5·6; N, 8·6; S, 9·8%.)

1-Dimethylaminonaphthalene-5-(N-benzyl)-sulphonamide (*IV*). Sulphonyl chloride (220 mg.) was dissolved in 2 ml. acetone and 0·5 ml. benzylamine was added. A white mass separated immediately. The mixture was taken to dryness and the residue dissolved in a little ethanol. On addition of water the sulphonamide crystallized. The pale-green needles were washed with water and recrystallized from 50% ethanol-water. Yield 190 mg., m.p. 139°. (Found: C, 66·5; H, 5·6; N, 8·3; S, 9·5. $C_{19}H_{20}O_2N_2S$ requires C, 67·0; H, 5·9; N, 8·2; S, 9·4%.)

Preparation of the conjugates

A weight of sulphonyl chloride equal to 1–2% of the protein was dissolved in 0·5 ml. acetone and added with stirring to 10 ml. of protein solution in 0·1 M-phosphate buffer, pH 7·5, or 1% $NaHCO_3$, kept at 0–3°. The reaction mixtures were left at 0–3° until the original turbid suspension cleared (5–12 hr.). The solutions were then centrifuged to separate suspended chloride, and dialysed with stirring in the cold against 0·2 M-KCl, or 0·15 M-K_2SO_4, with frequent changes of the latter until the outer liquid showed no appreciable fluorescence. (Excitation with Hg arc through Wood's filter.) This stage was reached with most proteins after about 2 days of dialysis. Serum albumin requires a considerably longer dialysis due to the adsorption of free dye. However, the adsorbed sulphonate could be easily separated by precipitation of the protein with ethanol. The conjugate was first dialysed against 0·1 M-acetate buffer pH 4·7, and an equal volume of 80% ethanol was added slowly at 0°. The precipitate was collected and washed repeatedly with 50% ethanol-acetate buffer at 0° until a sample of the supernatant showed very weak, polarized fluorescence (due to a trace of the conjugate in

solution). Finally, the precipitate was centrifuged and redissolved in cold 0·1 M-phosphate buffer pH 7·5, and dialysed against 0·2 M-KCl. One ethanol precipitation was usually enough to liberate all the adsorbed material though occasionally a second precipitation proved necessary. The combination of the sulphonyl chloride with the protein is a heterogeneous reaction, the yield of which largely depends upon the state of division of the sulphonyl chloride. If this is sufficiently fine a comparatively stable yellow suspension is obtained which, on reacting with the protein, becomes in the course of a few hours almost or completely transparent and much paler. If the initial suspension in 5% acetone water has a chloride content greater than about 0·2 mg./ml. some of it separates as crystals and the yield decreases. If the chloride content is kept below this limit and crystallization is avoided some 50–60% of it combines with the protein as judged by spectrophotometric measurements.

RESULTS

Absorption spectrum and fluorescence of 1-dimethylaminonaphthalene-5-sulphonic acid and derivatives

1-Dimethylaminonaphthalene-5-sulphonic acid and its sulphonamido derivatives show an absorption band in the 300–400 mμ. region. The maximum of this band is displaced towards the ultraviolet with increase in the ionic character of the —SO_2— group as shown in Table 1, but the molar extinction coefficient of the maximum varies very little in the different derivatives. The integrated area under the above absorption band was found to be constant within 5% in substances II–IV. On this basis it must be expected that τ_0 is essentially the same in all these cases (Lewis & Kasha, 1945). The absolute values of the lifetime of the fluorescence obtained from the equation given by these authors were $1{\cdot}1$–$1{\cdot}2 \times 10^{-8}$ sec., although for the reasons pointed out by Lewis & Kasha (1945) no more than an indication of magnitude should be expected from this figure.

Table 1. *Absorption spectra and limiting polarization of 1-dimethylaminonaphthalene-5-sulphonic acid and derivatives*

($p_{max.}$ is the polarization observed in glycerol solution at 3°. p_0 is the limiting polarization obtained by extrapolation from measurements in glycerol at several temperatures. The concentration of the solutions in glycerol was 3×10^{-3} g./l. The absorption spectra refer to solutions in water (I), 60% ethanol (II–IV) and absolute ethanol (V).)

Substance	Position of maximum (mμ.)	Absorption coefficient (cm.²/mol.) (×10⁶)	p_0	$p_{max.}$
I	312	4·55	0·250	0·245
II	329	4·05	0·267	0·255
III	340	4·40	0·267	0·260
IV	332	4·46	0·268	0·255
V	369	3·73	—	—

The fluorescence of the sulphonic acid and the substituted sulphonamides is quenched by acid, the region of rapid decrease of the fluorescence with pH being from 4 to 3. According to the theory of the quenching by collisions of the second kind (Wawilov, 1929) the quenching of a fluorescence with $\tau_0 \simeq 10^{-8}$ requires 0·1–0·01 M concentration of quencher. As this is about 50–100 times the hydrogen-ion concentration required, it may be concluded that the quenching is non-collisional. This is confirmed by measurements of the polarization of the fluorescence of acid-quenched solutions. The substances were dissolved in 1:2-dihydroxypropane, and dry hydrogen chloride gas was passed until a convenient degree of quenching was reached. The intensities before and after quenching (I_0 and I respectively) and the corresponding polarizations (p_i and p_q respectively) were recorded. The ratio of the lifetimes of the excited state before and after quenching τ_0/τ is (Sveshnikoff, 1936)

$$\frac{\tau_0}{\tau} = \frac{1/p_i - 1/p_0}{1/p_q - 1/p_0}.$$

Table 2 gives the values of I_0/I, p_i, p_q and τ_0/τ for substances I–IV. The small change in the polarization produced by the quenching reflects the long lifetime of the non-fluorescent form, the ratio Σ/τ_0 (Weber, 1948, 1950) being 50 to 100 in the different cases quoted in the table. This long-lived non-fluorescent form may be identified with the comparatively stable R-$N^+(CH_3)_2H$, the pK of which is in the neighbourhood of 4. The values of p_0, quoted in Table 1, were obtained by observation of the substances in glycerol at different temperatures.

Table 2. *Quenching of the fluorescence of 1-dimethylaminonaphthalene-5-sulphonic acid and derivatives*

(The concentration of the solutions was 6×10^{-3} g./l.)

	p_i	p_q	I_0/I	τ_0/τ
I	0·043	0·109	33	3·6
II	0·048	0·052	35	1·05
III	0·062	0·081	52	1·4
IV	0·053	0·070	60	1·4

The protein conjugates showed an absorption band in the 300–400 mμ. region separated from the absorption band of the protein by a well-defined minimum. Calculation of the number of molecules of sulphonamido derivative per molecule of protein has been based on the assumption that the molar absorption coefficient at the maximum of the conjugates is the same as in the sulphonamides described. An average of $4{\cdot}3 \times 10^6$ cm.²/g.mol. has been used.

A. Ovalbumin conjugates

The absorption spectrum of the conjugates is shown in Fig. 1. The maximum of the band due to the presence of the dimethylaminonaphthalene groups lies at 344 mμ. This is noticeably displaced towards the red compared with the bands of the serum albumin (maximum at 332 mμ.), and of the

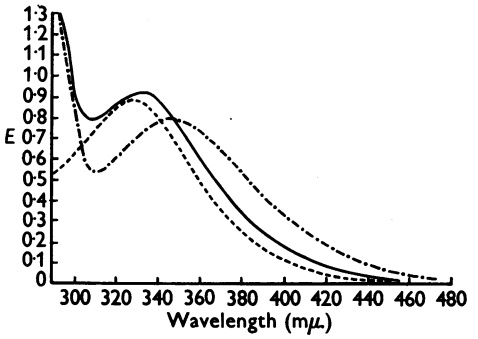

Fig. 1. Absorption spectra of conjugates. ——, Bovine serum albumin (protein concentration 0·65 %); –·–·–, ovalbumin (conjugate *B*) (protein concentration 0·48 %); - - - - - -, polylysine (concentration 0·5 %).

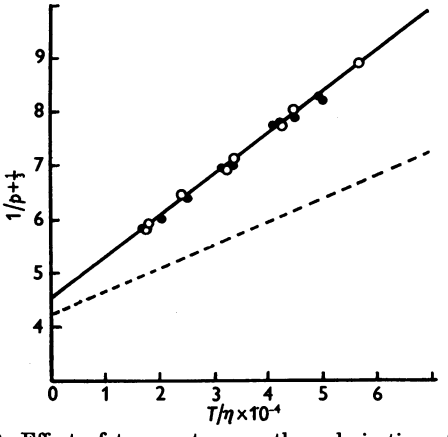

Fig. 2. Effect of temperature on the polarization of the fluorescence of ovalbumin conjugates. –○–○–, Conjugate *B*; –●–●–, conjugate *A*. The broken line is the slope observed with bovine serum albumin conjugates.

polylysine conjugate (maximum at 329 mμ.). This causes the solutions of ovalbumin conjugates to appear yellow while the serum albumin conjugates of similar concentration are colourless. The contents of naphthalenic groups calculated for the three conjugates studied were: $A = 1{\cdot}7$ mol. naphthalene/mol. protein; $B = 2{\cdot}0$; $C = 2{\cdot}4$. These resulted from reaction of about half the sulphonyl chloride present.

Polarization of the fluorescence

Effect of concentration. In theory, changes in the concentration of the emitting units cannot be expected to have any effect on the polarization. This was repeatedly confirmed by experiment. Solutions of ovalbumin conjugates in 0·05 M-phosphate buffer pH 7 showed no detectable change in the range of concentrations investigated, 1–0·05 % protein. Solutions of ovalbumin (0·6–1 %) dialysed against distilled water ultimately reached a pH of 4·85 and the recorded polarization was the same as with solutions in buffer, nor was this polarization changed by addition of 0·2 M-potassium chloride or by dilution down to 0·1 %.

Effect of temperature. The data are shown in Table 3 and in Fig. 2. They allow the conclusion that Perrin's law of depolarization is closely followed over the range of temperatures studied, namely 3–45°. In all the conjugates studied the plotting of $1/p$ against T/η yields straight lines with regression

Table 3. *Polarization of the fluorescence of ovalbumin conjugates at different temperatures*

Temp. (°)	T/η	p Observed	p Calculated
Conjugate *B*. Solvent: 0·04 M-phosphate buffer, pH 6·85. Protein concentration: 0·24 g./100 ml.			
4·0	176	0·182	0·180
14·8	243	0·163	0·165
25·9	341	0·148	0·147
37·5	451	0·131	0·131
35·3	429	0·135	0·134
48·5	571	0·117	0·117
24·4	328	0·149	0·151
4·5	180	0·178	0·180
Solvent: 0·1 M-NaOH. Same protein concentration			
3·0	170	0·181	0·182
12·1	231	0·171	0·168
26·5	346	0·146	0·147
Conjugate *C*. Solvent: 0·04 M-phosphate buffer, pH 6·85. Same protein concentration			
3·5	171	0·182	0·181
14·9	251	0·164	0·164
8·7	206	0·175	0·174
35·0	426	0·135	0·135
25·4	337	0·150	0·148
42·4	503	0·127	0·125
42·0	498	0·127	0·126
33·9	416	0·135	0·136
5·2	184	0·181	0·179
10·0	216	0·172	0·172
18·3	179	0·163	0·158
23·9	279	0·151	0·151
31·8	395	0·139	0·139
37·8	454	0·133	0·131

The polarizations have been calculated from the equation

$$\frac{1}{p} = 4{\cdot}22 + 0{\cdot}75 \times 10^{-4}\frac{T}{\eta}.$$

The measurements are given in the order in which they were performed.

Conjugate	$1/p_0$	$b \times 10^6$	$\beta \times 10^6$
A	4·19±0·06	76·7±1·8	18·2±0·5
B	4·20±0·06	75·6±1·8	17·9±0·5
C	4·24±0·05	75 ±1·3	17·7±0·4

coefficients with a standard error of about 2%. According to Eqn. 1 the regression coefficient b contains the factor $1/p \mp \frac{1}{3}$, so that for two conjugates having intercepts $1/p_{01}$ and $1/p_{02}$

$$\frac{\rho_2}{\rho_1} = \frac{b_1 \mid 1/p_{01} \pm \frac{1}{3}}{b_2 \mid 1/p_{02} \pm \frac{1}{3}}.$$

Therefore, if the linear law of depolarization is followed, the characteristic quantity for the conjugate is

$$\beta = \frac{b}{1/p_0 \pm \frac{1}{3}}.$$

This is given in Table 3 for the egg albumin conjugates together with the propagated error

$$\frac{\Delta\beta}{\beta} = \sqrt{\left\{\left(\frac{\Delta b}{b}\right)^2 + \left(\frac{\Delta(1/p_0 + \frac{1}{3})}{1/p_0 + \frac{1}{3}}\right)^2\right\}}.$$

The thermal effects were perfectly reversible. No detectable changes in the polarization were found after keeping the protein for 1 hr. at 45°.

The use of distilled water or dilute salt solutions from 2 to 50° allows a range of values of T/η between 1·6 and 6×10^4. To reach lower values of T/η, it is necessary to increase the viscosity of the solution by addition of a foreign substance. It is very doubtful whether the microscopic viscosity, which alone determines the resistance to the molecular rotations, can be equated in all cases with the viscosity measured by flow. To test this point the effect of electrolytes and of sucrose on the polarization was tried. Using sodium chloride and potassium chloride it was found that the increase in polarization produced by addition of electrolyte was much smaller than predicted by Eqn. 1. Concentrations below 1 M yielded polarization values which were indistinguishable from those in distilled water. Therefore in the calculation of T/η of solutions in dilute buffer ($M \leqslant 0{\cdot}2$) the viscosity of the pure solvent was used in every case. Better agreement with Eqn. 1 was observed when sucrose was used to increase the viscosity of the solvent particularly in the case of neutral solutions of serum albumin as described later in this paper. The viscosity of a 60% (w/v) sucrose solution at 3–5° is of the order of poises (Bingham, 1922). From Eqn. 1, if $3\tau_0/\rho_h$ in water is 1 or less, its value in 60% sucrose should be negligible compared to 1 and the polarization observed under this condition should not differ from p_0, as determined by extrapolation, if the linear law is followed throughout the range of T/η. Native ovalbumin conjugates dissolved in 60% sucrose (w/v) yielded $p = 0{\cdot}236 \pm 0{\cdot}004$, which is within the errors of the experiments the same as the extrapolated p_0. Therefore there is no detectable curvature and according to Eqn. I, 29,* the relaxation time calculated from the slope and intercept is ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoid. Consequently

$$\frac{\tau_0}{\rho_h} = \frac{1}{3}\,\frac{1/p - 1/p_0}{1/p_0 + \frac{1}{3}} = \frac{bT/3\eta}{1/p_0 + \frac{1}{3}} = \frac{\beta T}{3\eta}$$

from Eqn. 1. If $\beta = 1{\cdot}68 \times 10^{-5}$ and T/η at

$$25° = 3{\cdot}33 \times 10^4,$$

then $(\tau_0/\rho_h)_{25°} = 0{\cdot}186$. According to Oncley (1942), the harmonic mean of the principal relaxation times of the rotation of ovalbumin at 25° in water is $7{\cdot}55\ 10^{-8}$ sec. so that $\tau_0 = 1{\cdot}4 \times 10^{-8}$ sec. It will be convenient to assume in the future a value of $1{\cdot}4 \times 10^{-8}$ sec. for the lifetime of the excited state of the fluorescence of the conjugates. By introducing it in Eqn. 1 the relaxation times of the rotation of the conjugate molecules can be calculated, provided the depolarization follows the linear law.

Effect of pH *on the relaxation time of the rotation*

Changes of the pH of the solution between 1·5 and 13 produce no immediate changes in the polarization of the fluorescence. This is shown in Fig. 3

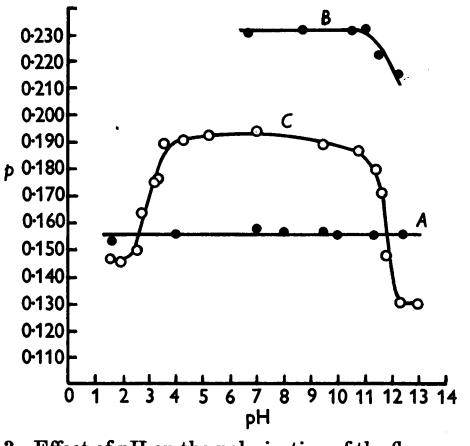

Fig. 3. Effect of pH on the polarization of the fluorescence. *A*, native ovalbumin; *B*, heat denatured ovalbumin; *C*, bovine serum albumin.

which refers to measurements done at 19°. The protein was dialysed against 0·02 M-potassium chloride and the pH adjusted, by the addition of hydrochloric acid or sodium hydroxide, to the required value which was measured by glass electrode. The polarization at each value was recorded as soon as possible afterwards, and the measurement repeated at intervals during at least 1 hr. During this time no change whatsoever was found in solutions between pH 3 and 13. At pH values between 1 and 3 a slow increase in the polarization was noticed. This increase was much faster if the solutions were kept at 37°. When the solutions kept for some time at pH 2 were adjusted to the isoelectric point a precipitate of

* This refers to Eqn. 29 of the first paper of this series (Weber, 1952). This notation is used throughout.

denatured protein appeared. The supernatant solution showed the polarization value corresponding to the native protein. This acid denaturation was studied more fully and the results are described below.

Time course of the acid denaturation

Fig. 4 shows the changes in the polarization during the course of the denaturation of 0·6% solutions of ovalbumin kept at pH 1·53–1·65. The protein was dialysed against distilled water, and then diluted to the required concentration. The

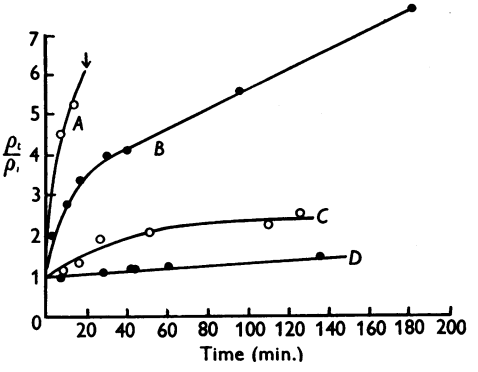

Fig. 4. Time course of the acid denaturation of ovalbumin. *A*, 0·2M-KCl, 30°; *B*, salt-free, 30°; *C*, 0·2M-KCl, 3°; *D*, salt-free, 3°.

polarization of the fluorescence was then determined at two temperatures, and finally the pH was adjusted to the required value by addition of M-hydrochloric acid. The cell was maintained at constant temperature (±0·5°) and readings were made at intervals during 2 hr. The ratio of the apparent relaxation time ρ_t to the initial relaxation time ρ_i is given by the simple equation

$$\frac{\rho_t}{\rho_i} = \frac{1/p_i - 1/p_0}{1/p_t - 1/p_0},$$

where p_i is the initial polarization and p_t its value at time t. It is assumed that the lifetime of the excited state does not change with pH. This is substantiated by the fact that in the protein solution kept at 3° in the absence of salt the value of p during the first 10 min. was the same as before the addition of the acid. In the other cases the course of the reaction shows that the value of p at zero time cannot differ greatly from that at neutral pH. Moreover, as shown by Table 2, the quenching of the fluorescence of several derivatives of 1-dimethylaminonaphthalene-5-sulphonamide by H ions does not result in any rapid change of the lifetime of the excited state of the fluorescence.

The curves of Fig. 4 show that the increase in the apparent relaxation time of the particles is much faster at the higher temperature, and faster in the presence than in the absence of salt. This applies also to the final values reached after several hours. At 30°, in the presence of salt (0·2M-potassium chloride), a copious precipitate occurred after a few minutes (arrow in Fig. 4) and no further measurements were possible.

Alkaline denaturation

The values quoted in Table 3 show that ovalbumin dissolved in 0·1M-sodium hydroxide had the same relaxation time of the rotation as at neutral pH. On adjusting the pH to the isoelectric point, the bulk of the protein separated. Therefore in alkaline solution, although the protein was denatured just as readily as in acid, no increase in the relaxation time was observed. When the isoelectric precipitate was redissolved in 0·1M-phosphate buffer, pH 7·8, considerable increase in the relaxation time of the rotation was observed. Even in 0·1M-sodium hydroxide the average relaxation time of the dissolved isoelectric precipitate was considerably higher than the native protein.

Solutions of ovalbumin kept at pH 12–13 for 24 hr. showed a decrease in the relaxation time, probably due to partial hydrolysis of the protein. Such decrease was never observed in experiments of short duration.

Urea denaturation of ovalbumin

To a solution of ovalbumin in distilled water (pH 4·9) was added 0·49 g. urea/ml. of original solution. After standing for 4 days at 0° the urea was dialysed away and the solution finally equilibrated against 0·05M-phosphate buffer, pH 7·3. The crystal clear solution had a concentration of 0·12% protein. Fig. 5 shows that the average relaxation time of the rotation of the particles had increased by about eight times as compared with that of the native protein. On heating the solution the polarization decreased as expected from the linear law.

Heat denaturation

Dilute solutions of ovalbumin (0·1–0·2%) in 0·05M-phosphate buffer, pH 7, boiled for a short time showed strong increase in the polarization of the fluorescence (Fig. 5). After boiling the solutions for 5 min. the absorption spectrum showed a maximum at 332 mμ. and was now practically coincident with the spectra of the serum albumin conjugates. If the solution was boiled for 1 min. a hybrid curve showing two maxima at 332 and 342 mμ. was obtained (Fig. 6).

The difference of absorption curves of native egg and serum albumins is not likely to be due to any difference of the covalent attachment of the sulphonamido groups. Since denatured ovalbumin, in common with other denatured proteins (Oster & Grimsson, 1949), resembles serum albumin (as

native egg albumin does not) in having a great capacity for adsorption of dyes, the identity of the absorption curve of their conjugates may be accounted for by secondary binding of the sulphonamido groups, which would be absent in conjugates

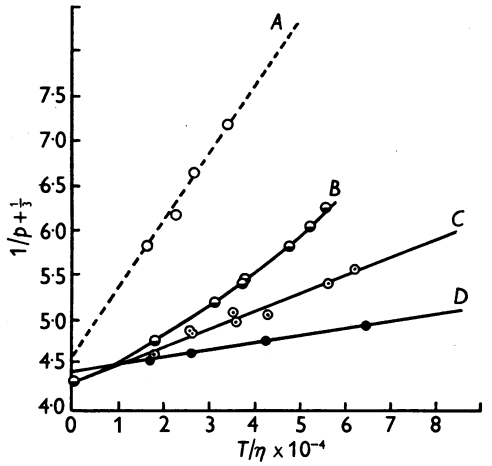

Fig. 5. Polarization of conjugates of denatured ovalbumin. *A*, ovalbumin denatured in alkali (dissolved in 0·1 M-NaOH); *B*, heat-denatured in 0·04 M-phosphate buffer, pH 7·2; *C*, denatured by urea (solvent: 0·04 M-phosphate buffer, pH 7·2); *D*, denatured by acid, salt-free, pH 1·7. The broken line is the slope corresponding to the native ovalbumin.

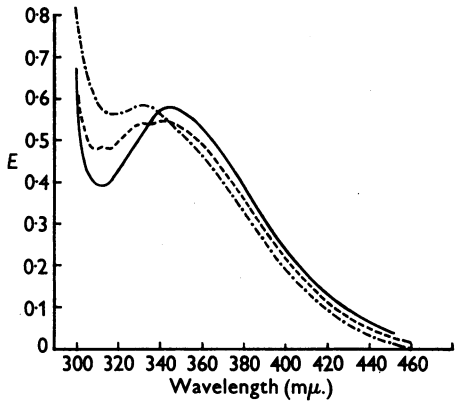

Fig. 6. Change of absorption spectrum of ovalbumin conjugates on heat denaturation. ——, native ovalbumin; - - - - - -, boiled for 1 min.; -·-·-·-, boiled for 5 min. Protein concentration 0·36 %.

of native ovalbumin. This is supported by the lower value of p_0 for native ovalbumin (0·236) than that for serum albumin (0·257), and the increase of the former value to $0{\cdot}254 \pm 0{\cdot}04$ on heat denaturation (measured in 60 % sucrose).

B. Bovine serum albumin conjugates

Absorption spectrum. The conjugates studied contained 1–3 mol. of naphthalene per mol. of protein of molecular weight 70 000. The absorption spectrum is shown in Fig. 1. The maximum of absorption is at 332 mμ., very nearly that obtained with a polylysine conjugate, though shifted slightly to longer wavelength. Such change has been found in the absorption spectra of many dyes adsorbed on serum albumin (Laurence, 1952). This is consistent with the secondary binding of covalently attached molecules suggested above. In the conjugate, the absorption spectrum of which is shown, there were 2·3 mol. of naphthalene per mol. of protein, resulting from the reaction of 60 % of the added chloride.

Effect of concentration on the polarization of the fluorescence. Solutions of 1–0·02 % showed no appreciable difference as regards the polarization of the fluorescence, both in distilled water (pH 5·25) and in 0·05 M-phosphate buffer, pH 7.

Temperature effect on the polarization. Solutions of conjugate at pH 6–8 followed the linear law between 3 and 50°. Above 50° a marked departure from linearity took place. The temperature effects were perfectly reversible. Keeping the protein solutions at this pH for 1 hr. at 59° did not result in any significant change in the observed polarization. Several conjugates prepared over a period of more than a year yielded entirely reproducible results. The regression coefficients of $1/p$ upon T/η varied from $4{\cdot}18 \times 10^{-5}$ to $4{\cdot}36 \times 10^{-5}$ in the different cases with an average of $4{\cdot}25 \pm 0{\cdot}1$. The value of $1/p_0$ was $3{\cdot}90 \pm 0{\cdot}06$. The data are given in Table 4 and in Fig. 7.

Polarization of the fluorescence in sucrose solutions. The data are shown in Table 4. It appears that the values of the polarization in 20 % (w/v) sucrose is what would be expected from Eqn. 1 if the viscosity measured by flow is introduced. The polarization calculated using $b = 4{\cdot}25 \times 10^{-5}$, obtained from measurements in water, agree with the observed values within the experimental error.

Fig. 8 gives the plot of $\dfrac{1/p + \frac{1}{3}}{1/p_0 + \frac{1}{3}}$ against τ_0/ρ_0; where ρ_0 is the relaxation time of a sphere of volume equal to that of the serum albumin molecule. If this has anhydrous molecular weight M and partial specific volume δ, and the hydration is H g. of water per g. of anhydrous protein

$$\rho_0 = \frac{3\eta}{RT} M (H + \delta).$$

Introducing $\tau_0 = 1{\cdot}4 \times 10^{-8}$ sec. from the measurements with ovalbumin $M = 7 \times 10^4$; $\delta = 0{\cdot}75$; and $H = 0{\cdot}15$ from the recent measurements of Haggis, Buchanan & Hasted (1951), we have

$$\frac{\tau_0}{\rho_0} = 6{\cdot}15 \times 10^{-6} \times \frac{T}{\eta}.$$

Table 4. *Polarization of the fluorescence of bovine serum albumin conjugate at different temperatures*

(Conjugate containing 2·3 mol. naphthalene/mol. protein. Solvent: 0·05 M-phosphate buffer, pH 6·77. Protein concentration: 0·12%.)

Temp. (°)	$T/\eta \times 10^{-2}$	p Observed	p Calculated
3·4	173	0·215	0·215
10·7	221	0·210	0·206
23·5	320	0·188	0·189
30·3	380	0·182	0·180
35·4	431	0·176	0·174
44·5	526	0·163	0·162
38·9	465	0·173	0·169
51·5	606	0·155	0·153
55·7	657	0·148	0·148
58·4	690	0·140	0·146
14·5	248	0·200	0·201
3·0	170	0·215	0·216
20·2	293	0·192	0·193
50·2	592	0·152	0·155
54·8	646	0·145	0·149
58·8	696	0·138	0·144
Same conjugate. Sucrose added to 20% (w/v).			
3·5	83	0·237	0·236
16·7	135	0·225	0·224
27·6	188	0·214	0·213
35·1	235	0·206	0·204
39·5	260	0·200	0·198
48·1	319	0·190	0·190
53·5	365	0·181	0·182

The polarizations have been calculated from the equation

$$\frac{1}{p} = 3{\cdot}90 + 0{\cdot}433 \times 10^{-4}\frac{T}{\eta}.$$

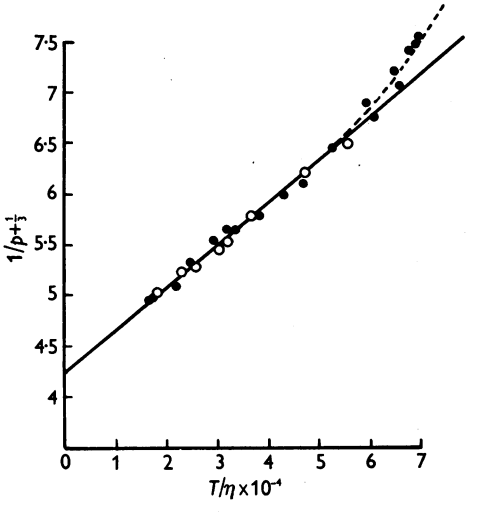

Fig. 7. The effect of temperature on the polarization of the fluorescence of bovine serum albumin conjugates. The open and filled circles correspond to two different preparations. The solid line is the regression line obtained from the observations at temperatures below 50°.

The figure shows that an axial ratio of 4 would account well for the observed values. If $H = 0{\cdot}3$ the axial ratio would be about 3 and 5 if $H = 0$.

The polarization recorded in 60% sucrose at 2° was $0{\cdot}257 \pm 0{\cdot}002$. Within the limits of the experimental error this is the same as the value obtained by extrapolation (0·256).

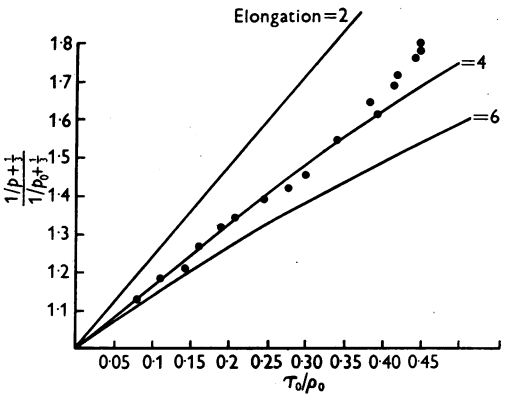

Fig. 8. Abscissa: τ_0/ρ_0. Ordinate: $\frac{1/p+\frac{1}{3}}{1/p_0+\frac{1}{3}}$, as explained in the text. The curves are the theoretical for ellipsoids of elongation 2, 4 and 6 (Weber, 1952).

The harmonic mean of the relaxation times of the rotation at 25° in water can be determined from Eqn. 1 in which $\tau_0 = 1{\cdot}4 \times 10^{-8}$ sec. We thus obtain $\rho_h = 1{\cdot}27 \times 10^{-7}$ sec., while Oncley (1942) gives $\rho_h = 1{\cdot}24 \times 10^{-7}$ sec. from measurements of the dielectric dispersion with horse serum albumin.

Observations on bovine serum albumin conjugate in acid solution

If a 0·1% solution of conjugate is thoroughly dialysed against distilled water and the pH adjusted to 1·8–2 by addition of a small amount of M-hydrochloric acid the polarization of the fluorescence drops from 0·196 (at 18°) to 0·147. Measurements at different temperatures at this pH yielded

$$b = (9{\cdot}68 \pm 0{\cdot}2) \times 10^{-5};$$
$$1/p_0 = 4{\cdot}08 \pm 0{\cdot}07;$$
$$\beta = (2{\cdot}2 \pm 0{\cdot}06) \times 10^{-5}.$$

In 60% sucrose $p = 0{\cdot}245$ in good agreement with the extrapolated p_0. The results are shown in Fig. 9. After neutralization by dialysis against 0·05 M-phosphate buffer, pH 7, the observed polarizations yielded $b = 4{\cdot}3 \times 10^{-5}$; $1/p_0 = 3{\cdot}95$; $\beta_0 = 1{\cdot}02 \times 10^{-5}$, showing that the changes in polarization with pH were perfectly reversible.

The intensity of the fluorescence of the salt-free conjugates at pH 2 was 60% of the intensity at pH 7, while the absorption spectrum showed no conspicuous change. The observations on the ovalbumin

conjugates, together with those on the quenching of the fluorescence of the sulphonamido derivatives, show that no change in the lifetime of the excited state is to be expected. It must be concluded that the changes in polarization are due to a change in the relaxation time of the rotation. In turn, this cannot be due to a simple change in shape of the serum albumin neutral molecule, if this is an ellipsoid of axial ratio 5 or less. On changing into a sphere, such

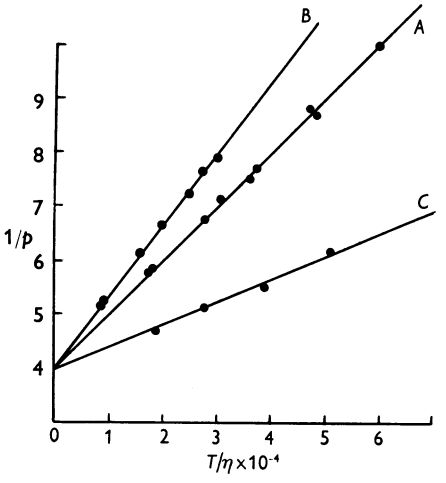

Fig. 9. *A*, polarizations of bovine serum albumin (0·12%) in salt-free pH 1·9 solution; *B*, 20% (w/v) sucrose at pH 1·9; *C*, solution *A* neutralized to pH 7.

an ellipsoid will have a relaxation time of 0·52 times the original value, while the observed ratio of β_0/β is 0·46. In order that this may be explained by a change in shape the neutral molecule would have to be an ellipsoid of elongation 8 or more or a flat molecule of axial ratio greater than 5. The absence of curvature in the plot of $1/p$ against T/η (Fig. 8) excludes the former, while the data of the dielectric dispersion (Oncley, 1942) do not support the latter. We may then conclude that molecular fission is the cause of the observed changes in the polarization. The number of resulting particles cannot be easily decided because of the unknown shape of the sub-units. If these are less asymmetric than the parent molecule their number cannot be other than two, but if their asymmetry is greater a larger number is possible. The fact that the resulting particles may not all be of equal size should, within wide limits, have little influence on the observed slope, provided that the original and resulting particles have similar shape. If we assume that the groups that can react with the sulphonyl chloride have equal affinity for this reagent, the particles will contribute to the total fluorescent intensity in proportion to the number of such groups that they possess. In the absence of more detailed information it is convenient to assume that, in a solution of particles that have reacted together with the sulphonyl chloride, f_i the contribution of each species to the total emitted intensity is proportional to its relaxation time. Therefore

$$f_i = \frac{N_i \rho_{hi}}{\sum_i N_i \rho_{hi}},$$

where N_i is the number of molecules of relaxation time ρ_{hi}. The weighted harmonic mean of the relaxation times is

$$\bar{\rho}_h = \frac{1}{\sum_i \frac{f_i}{\rho_{hi}}} = \frac{\sum_i N_i \rho_{hi}}{\sum_i N_i} = \bar{\rho}_a,$$

where $\bar{\rho}_a$ is the number average of the relaxation times. Thus, if the original and resulting particles have the same shape, the ratio of the β quantities is the number of units into which the original molecule has dissociated.

The polarizations obtained at pH 2 in 20% (w/v) sucrose (Fig. 9) were systematically lower than in water at equal values of T/η. The sucrose points fall evenly on a straight line with $b = 1{\cdot}30$; $1/p_0 = 4$; $\beta = 2{\cdot}70$. The difference from the values in water may be due to more complete dissociation in sucrose (hydrogen bonds?) or to differences between the microscopic viscosity and the viscosity measured by flow. It may be pointed out that at pH 2 a small percentage of the sucrose present was hydrolysed and therefore a chemical reaction of the hexoses with the protein cannot be excluded.

Effect of salt on the acid dissociation. In the presence of M-potassium chloride, or 0·1 M-potassium sulphate the dissociation did not take place when the pH was brought from neutrality to 1·9. If the salt was added after the dissociation was obtained, complete reversal was observed, the polarization increasing to the original value in neutral solution.

Observations on bovine serum albumin in alkaline solution

0·5 ml. of 0·5% salt free conjugate was mixed with 2 ml. of 0·1 M-sodium hydroxide and the polarizations at different temperatures recorded. When measurements at temperatures higher than 25–30° were attempted an irreversible fall in the polarization was noticed. This was also noticed at lower temperatures if the alkali was left to act for a longer time (i.e. 12 hr. at room temperature). The polarization measurements below 28° were temperature reversible and could be repeated with several conjugates within 5%. As shown in Fig. 10, these measurements yielded $b = 1{\cdot}19$; $1/p_0 = 4{\cdot}34$; $\beta = 2{\cdot}56$. The polarization in 60% sucrose was 0·230 in excellent agreement with the extrapolated value.

Solutions left for an hour at 18°, and then neutralized, showed that the dissociation in alkali is

completely reversible as regards the relaxation time of the rotation. Solutions left for 10 hr. in alkali at 4° showed when neutralized a fall in the polarization indicating that if the action of the alkali is prolonged the reaction is no longer wholly reversible.

The slope and intercept of the solutions in alkali show differences from the values in acid. The comparatively minor differences in the values of β (0·46 and 0·40 respectively) can be due to a variety of causes, the analysis of which is not possible with the sole resources of this method. As regards the decrease in p_0 observed in alkali, several sources (Klotz, 1949; Laurence, 1952) indicate that the binding power of serum albumin is much diminished in alkaline solution. It may then be expected that

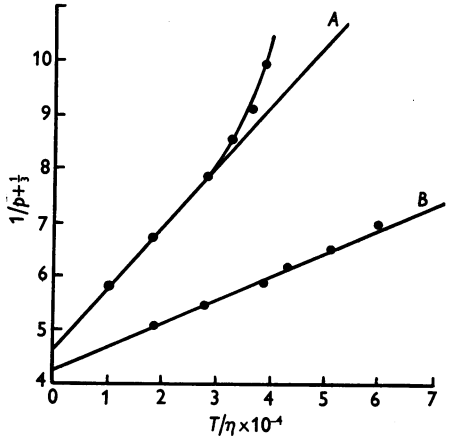

Fig. 10. *A*, polarizations of bovine serum albumin in 0·1 M-NaOH; *B*, a similar solution, kept for an hour at room temperature and then neutralized.

the freedom of rotation of the attached molecule should be larger in the latter case, leading to a decrease in the limiting polarization (Weber, 1952). The alkaline dissociation is largely unaffected by salt.

Effect of pH *on the relaxation time of the rotation*

Fig. 3 shows the effect of pH on the polarization of the fluorescence of 0·1% salt-free conjugates. The pH changes were obtained by addition of dilute hydrochloric acid or sodium hydroxide respectively and measured by glass electrode. The following points were noticed: (i) The shift in the polarization of the fluorescence with pH was complete in a matter of minutes, this being the time necessary to take a series of readings. In no case could a time effect be detected, even in solutions kept at 3°. (ii) Threefold dilution at any pH value did not result in any conspicuous change in the polarization. (iii) The main changes took place between pH 4 and 2·5 on the acid side, between 9·5 and 11·8 on the alkaline side. No further change was observed below pH 2 or above pH 12. (iv) The neutralized solutions showed no detectable difference from the original neutral solution. The most important fact to be noticed here is the complete correspondence of the regions of rapid change of the polarization with the main regions of titration of the protein.

Effect of urea treatment of the serum albumin conjugates

After treatment with 6 M-urea and subsequent removal of the latter by dialysis, the plot of $1/p$ against T/η, as shown in Fig. 11, gave a slope of

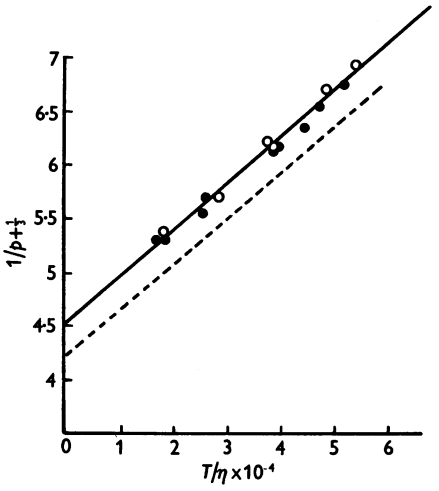

Fig. 11. Bovine serum albumin regenerated from 6 M-urea. The filled and open circles correspond to two independent experiments. The broken line is the slope of the native albumin.

0·40–0·42 in the different preparations, and intercept varying from 4·28 to 4·10. The slope was therefore not significantly different from that of the native protein, while the intercept was slightly, though consistently, higher. In 60% sucrose the observed polarization was $0·245 \pm 0·005$. The protein regenerated from urea has been found by sedimentation studies (Putnam, Erickson, Volkin & Neurath, 1943) to have a molecular weight equal to the native, and the observations here reported agree with it. The increase in the intercept can be interpreted as an increase in the rotational freedom of the coupled molecule due to decrease in the binding power of the protein, and a study of this property in urea-regenerated serum albumin may decide on the validity of this interpretation.

DISCUSSION

As described in the experiments, the sulphonyl chloride reacted very readily with the primary amino groups so that the formation of sulphon-

amido linkages as the most important product of the reaction with the protein offers little doubt. The absorption spectrum of the sulphonyl chloride presents a maximum at 369 mμ. and therefore, if any important amount of unreacted chloride were to remain adsorbed to the protein, a second maximum or an inflexion should appear in that region of the absorption spectrum of the conjugates. Such inflexion was observed in mixtures of proteins and chloride at the beginning of the reaction, but as this proceeded the inflexion decreased and disappeared.

Perrin's law of depolarization is followed by the serum albumin and ovalbumin conjugates within the limits of the experimental error. If the validity of Eqn. I, 28 is accepted, the absence of visible curvature may be taken as an indication that the molecules are flat or only moderately elongated. In all cases where the straight line law was followed excellent agreement was found between the limiting polarization p_0 as determined by extrapolation and the polarization observed in 60% sucrose solution. The differences in the value of p_0 in the different conjugates may be attributed to the varying degree of rotation about its conjugating bond allowed to the coupled molecule. The calculation of absolute values of the mean relaxation time of the rotation requires τ_0 to be known. Relative values of ρ_h may, however, be calculated if τ_0 is assumed to be the same in the different conjugates. This can only be decided by direct measurement of τ_0, but the data presented show that the calculation of this quantity using the available figures for the relaxation times of the rotation of ovalbumin and serum albumin leads in both cases to the same value, namely $\tau_0 = 1{\cdot}4 \times 10^{-8}$ sec.

It is also assumed that no change takes place in the lifetime of the excited state between pH 1·5 and 14. Ovalbumin conjugates give the same polarization between these extreme values provided the observations in acid are done rapidly and at low temperature to avoid denaturation. The conjugates at pH 1–2 show a decrease in the fluorescence intensity and the lack of change in the lifetime of the excited state must be attributed to the long lifetime of the non-fluorescent form (Weber, 1948). A study of the quenching of the fluorescence of the sulphonamido derivatives of 1-dimethylaminonaphthalene-5-sulphonic acid makes clear that such is the case and that moderate degrees of quenching should not alter sensibly the value of τ_0. It is worthy of note that the quenching of the substituted sulphonamides took place at pH 3–4, while the conjugates showed little quenching (about 30%) at pH 1·5. This difference is readily explained by the fact that at pH lower than the isoelectric point the ionic atmosphere of the protein will contain fewer hydrogen ions than the bulk of the solvent. The fluorescent intensity of the conjugates of serum albumin was considerably greater than those of native ovalbumin. Here once more the notion of a tautomeric non-fluorescent modification endowed with long life is probably valid.

Ovalbumin conjugates. The most important fact to emerge from the study of these conjugates is that reversible dissociation of the protein, by acid, alkali, or heat, was never seen and that the relaxation time increased considerably on denaturation by acid, alkali, heat, or urea. This increase in the observed relaxation time cannot be explained by an increase in the axial ratio of the particles alone. If the original quasi-spherical ovalbumin molecule is drawn into an ellipsoid of infinite axial ratio, Eqn. I, 28 shows that the apparent relaxation time cannot increase by a factor greater than 3. In the experiments, however, as shown in Figs. 4 and 5, increases of eight times and more were observed. These results lend themselves to a simple and complete interpretation if aggregation of denatured molecules is assumed to be the main cause of the increase in the relaxation time of the rotation. In the experiments on the time course of the acid denaturation, a visible precipitate appeared only in the case where the rate of increase of the relaxation time was a maximum, suggesting a continuous transition between the postulated aggregates and the precipitate. The isoelectric precipitation of denatured ovalbumin may be considered as the continuation of the aggregation on to a macroscopic scale.

If the aggregation is due to interactions which, like hydrogen bonds and van der Waals forces, decay rapidly with the distance, the main factor determining the growth of the particles will be the extent to which the electrostatic repulsion between the molecules can offset the thermal agitation and thus prevent the approach and interaction that leads to permanent binding (Verwey & Overbeek, 1948). The addition of electrolyte by decreasing the thickness of the double layer lets the particles grow larger and in the experiment at 30° the co-operative effect of salt and temperature allowed indefinite aggregation and flocculation to take place. The acid denaturation was accompanied by immediate increase in the relaxation time, while the protein denatured by alkali did not show it until the pH was brought near the isoelectric point. Moreover, when dissolved in acid, the alkaline denatured ovalbumin showed also increased relaxation time. A likely explanation of these facts is that the aggregation in acid is due to hydrogen bonds mainly from the uncharged carboxyl groups. No comparable source of hydrogen bonds exists in the alkaline protein.

The aggregation of ovalbumin in acid has been shown by Huang & Wu (1930) by osmotic pressure measurements.

Serum albumin conjugates. In contradistinction to ovalbumin the relaxation time of the rotation showed marked dependence on the pH of the solutions. The data presented show that the changes cannot be attributed to increase in τ_0 or to a simple change in shape of the serum albumin molecules, and that reversible dissociation into sub-units must be admitted. The study of this dissociation will be the object of another paper. It need only be indicated here that the departure from the linear law above 50° observed in neutral solution (Figs. 7 and 8) can be explained by thermal dissociation of the molecule.

SUMMARY

1. The preparation of stable fluorescent conjugates of ovalbumin and bovine serum albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride is described.

2. It is shown that the fluorescence of solutions of the purified conjugates follow Perrin's law of depolarization, yielding straight lines when the reciprocal of the polarization is plotted against the ratio of the absolute temperature to the viscosity of the solvent.

3. The slopes of the conjugates studied are reproducible within 2%, and for ovalbumin and bovine serum albumin at neutral pH are in the ratio of the harmonic mean of the principal relaxation times of the rotation as required by theory.

4. The polarizations observed in 60% sucrose solution are, in all cases studied, in excellent agreement with the limiting polarization obtained by extrapolation.

5. The relaxation time of the rotation of ovalbumin is shown to be independent of pH within a wide range (pH 1·5–14).

6. The denaturation of ovalbumin by acid, urea or heat results invariably in an increase in the relaxation time of the particles. The effect of temperature and of neutral salts on the acid denaturation has been studied.

7. The polarization of the fluorescence of bovine serum albumin conjugates is independent of pH between 4 and 9, but falls rapidly outside this region to reach lower stable values at pH 2 and 12 respectively. It is shown that only reversible dissociation into sub-units can explain these facts.

8. Bovine serum albumin regenerated from urea does not differ appreciably from the native species as regards particle size.

9. The preparation and characterization of 1-dimethylaminonaphthalene-5-sulphonyl chloride and of several sulphonamido derivatives are described.

The author is indebted to Dr Malcolm Dixon, F.R.S., under whose supervision this work was begun, for many suggestions and criticisms. He wishes also to express his gratitude to Dr K. Bailey and Dr F. Sanger, and to Mr D. J. R. Laurence and Mr T. C. Tsao for many interesting discussions. The micro-analyses have been performed by Weiler and Strauss, Oxford.

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