A long series of studies has shown that serum albumin changes very markedly, though reversibly, certain of its physicochemical properties over the pH range 4 to 2 (1). The sedimentation constant (1), translational diffusion coefficient (2), rotational relaxation time (3, 4), and specific viscosity and specific optical rotation (5) all show changes of considerable magnitude. To this list low angle x-ray scattering has been added recently (6).

From the data of sedimentation and diffusion, as well as light scattering (2), the molecular weight at neutral pH of 68,000 is known to remain unchanged in acid solution. Inspection of the values of Table I with this fact in mind shows that, whereas the frictional coefficient for the translation increases in acid solution—as revealed by a decrease of both s and D—the average frictional coefficient for the rotation calculated from the mean rotational relaxation time, \( \rho_h \), decreases by a factor of 2. A model that would account for such changes is one in which the protein is composed of globular parts linked by flexible peptide chain segments. The addition of acid is supposed to produce, through electrostatic repulsion, a spatial separation of the globular parts, allowed in this protein because of the flexible links between them. A similar change would be expected to occur in alkaline solution and has indeed been experimentally demonstrated (3).

An over-all swelling of the molecule would give rise to the observed decrease in s and D but should lead to an increase in \( \rho_h \), as observed by Churchich (7) in reduced bovine serum albumin and by Wahl (8) in synthetic random coil polymers. A model of globular and flexible parts, or one closely equivalent, has been proposed by Luzzatti, Witz, and Nicolaieff (6) from observations of low angle x-ray scattering alone.

An almost inescapable consequence of this model is that a protease acting in acid solution should preferentially act upon the peptide bonds of the linking segments, thus liberating a small number of globular units.

This paper describes experiments that show that a short digestion with pepsin produces the appearance of a small number of fragments with high molecular weight, the supposed compact units present in the molecule, and describes the properties of isolated fragments of digestion are described.

EXPERIMENTAL PROCEDURE

Crystalline bovine serum albumin (Armour and Company) was used throughout.

Crystalline pepsin (Worthington Biochemical Corporation) was used. An absorbance of 1 liter g\(^{-1}\) cm\(^{-1}\) at 280 nm was used in calculating the amount of pepsin in solution.

The magnesium salt of 1-anilinonaphthalene-8-sulfonic acid was recrystallized several times from water after filtration of the hot solutions successively through charcoal and talc. The original material was Eastman Kodak 1-anilinonaphthalene-8-sulfonic acid. The green crystals of the magnesium salt were dried at 120° for several hours. Their molar absorption coefficient was observed to be independent of concentration in the range 5 \( \times \) 10\(^{-3}\) to 5 \( \times \) 10\(^{-4}\) M. It was 4.95 \( \pm \) 0.1 \( \times \) 10\(^{8}\) cm\(^{-1}\) atm\(^{-1}\) at 350 mμ.

The measurements of fluorescence polarization were done in the apparatus described by Weber (9). Relative fluorescence efficiencies were determined by measuring the photocurrents with a sensitive galvanometer. In either case the fluorescence was excited by the 365-μm line isolated from a high pressure mercury arc by a filter (Corning Glass 7-60). The fluorescent light was filtered through a 2-mm layer of a molar solution of NaNO\(_2\) and a 3:1 Corning glass filter. The light detectors were EM1 9558 photomultipliers.

The measurements were done at 25°. To increase the viscosity of the solutions, sucrose in known amount was added, and the viscosity at 25° was obtained by interpolation based on the values of Bingham and Jackson (10).

RESULTS

I. Experiments with Conjugates of Bovine Serum Albumin with Dimethylaminonaphthalenesulfochloride

The changes in rotational relaxation time obtained in acid solutions of bovine serum albumin may be conveniently followed by the decrease in the polarization of the fluorescence of conjugates of albumin with dimethylaminonaphthalenesulfochloride. With the experimental technique utilized (10), the polarization of the fluorescence of 10\(^{-4}\) M solutions of conjugates with 2 moles of naphthyl residue per mole of albumin can be measured in a few minutes with a precision of ±1%. The conjugates employed contained 1.5 to 2 naphthyl residues per mole of BSA. They were prepared, and the absence of free dimethylaminonaphthalenesulfonic acid was ascertained, as already described (3, 11).

To follow the digestion of DNS-BSA by pepsin, samples of the incubation mixture were withdrawn at intervals and rapidly
diluted with 0.1 m phosphate buffer, pH 7.5. At this pH the pepsin digestion was stopped while the dilution was intended to render negligible any concentration-dependent aggregation of the digestion products. The results presented below show that such aggregation was present in our case and that dilution was indispensable to overcome it. All the polarization values quoted in the tables and figures refer, unless otherwise stated, to solutions of protein concentration of 0.04 to 0.01% (6 × 10⁻⁶ m to 1.5 × 10⁻⁶ m DNS-BSA).

Time Course of First Stages of Pepsin Digestion—Fig. 1 shows the time course of the digestion of 1.7% DNA-BSA by pepsin (molar ratio of pepsin to DNS-BSA, 1:400) at pH 2.0 and 25°. The closed circles refer to observations in 0.1 m phosphate buffer, pH 7.5; the open circles, to samples of incubation mixture diluted with 0.1 m citrate-phosphate buffer, pH 2.0. Observations of the latter were made as rapidly as consistent with good accuracy to render minimal the slow change in polarization caused by the continuing digestion. The difference in the polarization ordinate between the two curves at any given time is a measure of the "acid effect." This difference is seen to decrease as the digestion proceeds and to disappear after 15 minutes. As Fig. 1 shows, a first rapid fall in the polarization is followed by a phase of much slower change. On the model suggested in the introduction, the rapid phase may be expected to result from liberation of the globular fragments and the second slower phase from the further digestion of such fragments.

A reaction velocity may be defined as follows. The first stages of pepsin digestion are assumed to result in the uniform appearance of digestion fragments yielding, when alone in solution, a polarization, \( p_f \), at the expense of the original DNS-BSA, which by itself has polarization, \( p_i \). The observed polarization \( p(t) \) at any time, \( t \), during the rapid phase is

\[
p(t) = c(t) p_f + \left(1 - \frac{c(t)}{c_0}\right) p_i \tag{1}
\]

where \( c(t) \) is the concentration of protein converted into fragments at time \( t \), and \( c_0 \), the original or initial DNS-BSA concentration. The initial reaction velocity, \( V_i \), is

\[
V_i = \left( \frac{dc(t)}{dt} \right)_{t=0} = \left( \frac{dp(t)}{dt} \right)_{t=0} \left( \frac{c_0}{p_f - p_i} \right) \tag{2}
\]

From the last equation it is seen that one can define an absolute reaction velocity, in moles of DNS-BSA digested per mole of pepsin from the initial slope in the plot of \( p \) against \( t \), on condition of determining independently the value of \( p_f \) due to the fragments alone. The presence of a second slow phase permits us to fix this value, independently of the pepsin-albumin ratio, absolute albumin concentration, and pH (in the range 1.9 to 3.25) of the original incubation mixture, at \( p_f = 0.192 \pm 0.005 \). Since \( p_i = 0.310 \pm 0.003 \), this results in an error in \( V_i \) of \( \pm 8% \), a precision sufficient for our present purpose.

Dependence of \( V_i \) upon DNS-BSA and Pepsin Concentration—Table II shows \( V_i \) values for a series of DNS-BSA concentrations.

### Table I

<table>
<thead>
<tr>
<th>Hydrodynamic constants of bovine serum albumin in neutral and acid solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image_url" alt="Image" /></td>
</tr>
</tbody>
</table>

### Table II

Dependence of initial reaction velocity, \( V_i \), upon pepsin and DNS-BSA concentrations

<table>
<thead>
<tr>
<th>DNS-BSA</th>
<th>Pepsin</th>
<th>Molar ratio of DNS-BSA to pepsin</th>
<th>( V_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.016</td>
<td>94</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>0.016</td>
<td>138</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>0.016</td>
<td>282</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>0.016</td>
<td>370</td>
<td>32</td>
</tr>
<tr>
<td>27</td>
<td>0.050</td>
<td>270</td>
<td>34</td>
</tr>
<tr>
<td>27</td>
<td>0.075</td>
<td>180</td>
<td>30</td>
</tr>
<tr>
<td>36</td>
<td>0.050</td>
<td>360</td>
<td>30</td>
</tr>
<tr>
<td>36</td>
<td>0.065</td>
<td>275</td>
<td>27</td>
</tr>
</tbody>
</table>

Fig. 1. Time course of fluorescence polarization of DNS-BSA during pepsin digestion. DNS-BSA concentration was 17 mg per ml. Pepsin concentration was 20 mg per ml. The mixture was incubated at 25° in 0.2 m citrate-phosphate buffer, pH 2.0. - - - , Observations of incubation mixture diluted with 80 volumes of 0.1 m phosphate buffer, pH 7.5. O --- O, Observations of incubation mixture diluted with 80 volumes of 0.1 m citrate-phosphate buffer, pH 2.0.
and molar ratios of DNS-BSA to pepsin. For concentrations of DNS-BSA ranging from 0.3 to 3.6%, and molar ratios of DNS-BSA to pepsin of 94 to 375, $v_i$ remains almost constant—at 27 to 35 moles of DNS-BSA transformed per mole of pepsin per minute—showing that under these conditions maximal velocity is reached. It will be shown in the following paper that three or four fragments appear from the first stages of digestion, so that the observed velocity corresponds to a turnover number of 60 to 100 peptide bonds broken per mole of pepsin per minute, a figure comparable to the rate of hydrolysis of synthetic peptides by pepsin.

**Dependence of $v_i$ upon pH**—The results are summarized in Fig. 2, which shows the time course of the polarization changes for 1.8% DNS-BSA solutions digested by pepsin at a molar ratio of DNS-BSA to pepsin of 1:300, at various pH values from 1.9 to 4.05. The curves of pH values 1.9 to 3.25 are remarkably alike, and they yield virtually the same $v_i$ value. At pH 3.75, $v_i$ decreases to $rac{1}{2}$ of the low pH value, and at pH 4.05 it is only $2 \times 10^{-4}$ of it. These effects may be understood by reference to Fig. 3, which shows the curve of pH versus polarization for intact DNS-BSA (closed circles). The fact that the acid changes are only observable at pH 4 explains the “switch off” of the pepsin action at pH 4.05, if pepsin digestion is only possible in molecules that have undergone the changes observable at the acid pH values. The remarkable constancy of $v_i$ in the range 3.25 to 1.9 may be due to compensation of two opposing influences: on the one hand, the number of molecules in the configuration required for the pepsin digestion, which increases with decreasing pH; and on the other, an intrinsic rate of hydrolysis of the exposed peptide bonds, which, below pH 3.25, decreases with pH if its own pH optimum is about 3 or higher.

Alternatively the changes in the protein structure occurring below pH 3.25 may be irrelevant with respect to the action of pepsin on DNS-BSA, although leading to observable changes in polarization.

**Properties of pH 3.0 Initial Digest**—This is defined as the result of incubation of DNS-BSA and pepsin at 25°, in 0.2 M citrate-phosphate buffer, pH 3.0, for a time sufficient to bring the polarization of the fluorescence excited by 365-nm light to 0.193, when the polarization measurements are carried out at 25°, in 0.1 M phosphate buffer at a protein concentration of 0.1 mg per ml.

**Concentration Dependence of Fluorescence Polarization and Sedimentation Constant of Initial Digest**—Fig. 4 shows the dependence of the fluorescence polarization upon the protein concentration of the solution of digest observed. Since the polarization of intact DNS-BSA shows no concentration dependence whatever and the same is true of a variety of monodisperse systems, the observed concentration dependence must result from a change in the properties of the protein resulting from pepsin digestion. These changes are sufficiently prominent to cause the polarization of intact DNS-BSA to be about 0.5 times that of a monodisperse system, and the polarization of the digest solutions to be about 10 times that of intact DNS-BSA.

The concentration dependence of the fluorescence polarization of pH 3.0 initial peptic digest of DNS-BSA is shown in Fig. 4. The concentration dependence of the fluorescence polarization is given by the equation:

$$P = P_0 \left(1 - \frac{K}{[DNA]} \right)$$

where $P$ is the observed polarization, $P_0$ is the polarization of the undigested protein, $K$ is a constant, and $[DNA]$ is the concentration of DNS-BSA. The value of $K$ is obtained from the slope of the regression line of the data points, which yields $K = 1.2 \times 10^{-3}$.

**Concentration Dependence of Sedimentation Constant**—The sedimentation constant, $S$, of the digest solution is given by the equation:

$$S = S_0 \left(1 + \frac{K}{[DNA]} \right)$$

where $S_0$ is the sedimentation constant of the undigested protein. The value of $S_0$ is obtained from the slope of the regression line of the data points, which yields $S_0 = 3.2 \times 10^{-3}$.

Fig. 3. Effect of pH upon original DNS-BSA and DNS-BSA digest. DNS-BSA (open circles) or DNS-BSA initial digest (closed circles) (0.2 mg of DNS-BSA per ml, pH 3.0, were adjusted to the required pH with 1 N HCl).

Fig. 4. Concentration dependence of polarization of fluorescence of pH 3.0 initial peptic digest of DNS-BSA. [NRA] = Molar DNS-BSA concentration in original digestion mixture. Observations were made at 2° and 25° in 0.05 M phosphate buffer, pH 5.0.

While there was little difference in the values of $v_i$ at pH 3.5 or lower in different DNS-BSA preparations, one was observed in which digestion at pH 4.0 proceeded some 10 times faster than here indicated, although to a limited extent. This phenomenon is now under study.
TABLE III
Dependence of sedimentation coefficient upon protein concentration in initial digests of BSA by pepsin

<table>
<thead>
<tr>
<th>Protein concentration (g/100 ml)</th>
<th>s20w</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.85</td>
<td>2.33</td>
</tr>
<tr>
<td>0.42</td>
<td>2.18</td>
</tr>
<tr>
<td>0.21</td>
<td>1.98</td>
</tr>
</tbody>
</table>

TABLE IV
Dependence of fluorescence polarization upon ionic strength of solutions of initial digests of DNS-BSA

<table>
<thead>
<tr>
<th>Molarity of added KCl</th>
<th>Polarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75% DNS-BSA in 0.05 M phosphate, pH 7.0</td>
<td>0.228</td>
</tr>
<tr>
<td>0.1</td>
<td>0.215</td>
</tr>
<tr>
<td>0.5</td>
<td>0.210</td>
</tr>
<tr>
<td>1.0</td>
<td>0.207</td>
</tr>
<tr>
<td>2.0</td>
<td>0.205</td>
</tr>
<tr>
<td>0.001% DNS-BSA in 0.05 M phosphate, pH 7.0</td>
<td>0.199</td>
</tr>
</tbody>
</table>

Fig. 5. Sedimentation pattern of original BSA (upper) and BSA digested with pepsin at pH 3 for 20 minutes (lower). 1, 50 minutes after centrifuge reached maximal speed; 2, after 74 minutes; 3, after 90 minutes.

The polarization of the fluorescence of DNS-BSA gives a very convenient way of following the first stages of its peptic digestion. Since the experiments are easily reproduced, and it appears reasonable to assume that the 2 additional naphthyl residues do not introduce appreciable differences in the digestion of DNS-BSA as compared with unlabeled albumin, BSA can in principle be digested to a known extent by simple duplication of the conditions determined for DNS-BSA.

Ultracentrifugation Experiments—When 2% BSA was digested with 15 pg molar proportions of pepsin at pH 3.0 and aliquots were removed at various times of incubation, a single peak was observed in the sedimentation pattern in every case. Asymmetry of the trailing edge of the boundary became more marked as digestion proceeded, but in no case could a second, independent component be ascertained. Fig. 5 shows 1% protein solutions at zero time of incubation and after 15 minutes. The migration of the fragments as a single unit, already detected in the experiments with DNS-BSA, is also apparent in intact BSA. Fig. 6 shows graphically the plot of the sedimentation constant against time of incubation. Although it should be possible in principle to determine the extent of initial protein digestion by sedimentation studies, this is clearly an inconvenient method, that is much too cumbersome and time consuming. A more satisfactory way from aggregation of the digestion products. No time effects were observed upon dilution, the final value being reached within the time required to make the observations (1 to 2 minutes). The figure indicates that over the range of protein concentration of 1% to 0.1%, a rapid change in particle size with concentration takes place, a fact that may also be demonstrated by a sedimentation study of the digests. Table III gives the values of $s_{20w}$ measured with the synthetic boundary technique. The decrease in the $s_{20w}$ value with concentration is contrary to the usual increase shown by DNS-BSA, among other proteins. In each case a single peak was observed. The boundaries remained symmetrical during sedimentation, indicating a very rapid equilibrium between free and aggregated fragments.

The effect of ionic strength upon the polarization is seen in Table IV, which shows that at high ionic strength disaggregation takes place, the polarization of the fluorescence of a 0.75% DNS-BSA in 2 M KCl differing only a little from the value observed after 50-fold dilution without addition of salt.

II. Experiments with Intact Bovine Serum Albumin

The polarization of the fluorescence of DNS-BSA gives a very convenient way of following the first stages of its peptic digestion. Since the experiments are easily reproduced, and it appears reasonable to assume that the 2 additional naphthyl residues do not introduce appreciable differences in the digestion of DNS-BSA as compared with unlabeled albumin, BSA can in principle be digested to a known extent by simple duplication of the conditions determined for DNS-BSA.

Ultracentrifugation Experiments—When 2% BSA was digested with 15 pg molar proportions of pepsin at pH 3.0 and aliquots were removed at various times of incubation, a single peak was observed in the sedimentation pattern in every case. Asymmetry of the trailing edge of the boundary became more marked as digestion proceeded, but in no case could a second, independent component be ascertained. Fig. 5 shows 1% protein solutions at zero time of incubation and after 15 minutes. The migration of the fragments as a single unit, already detected in the experiments with DNS-BSA, is also apparent in intact BSA. Fig. 6 shows graphically the plot of the sedimentation constant against time of incubation. Although it should be possible in principle to determine the extent of initial protein digestion by sedimentation studies, this is clearly an inconvenient method, that is much too cumbersome and time consuming. A more satisfactory way

Fig. 6. Plot of $s_{20w}$ and $F/F_0$ against time of incubation at 25° of solutions of BSA (20 mg per ml) and pepsin (15 pg per ml). $F/F_0$ is the relative fluorescence efficiency of solutions containing 0.05 ml of incubation mixture (1 mg of BSA) in 3 ml of $2.2 \times 10^{-4} M$ ANS in 0.1 M buffer, pH 7.5.
of following the progressive fragmentation of the protein consists of measuring the changes in binding power towards the aromatic anion, 1-anilinonaphthalene-8-sulfonate.

**Experiments with ANS**—Weber and Laurence (12) found that a series of dyes, including 1-anilinonaphthalene-8-sulfonic acid (ANS), that exhibit in water solution very small quantum yields increase these dramatically upon adsorption on native serum albumin, although other proteins are virtually inactive. This property forms the basis of the method of determination of albumin in plasma of Fildes, Laurence, and Rees (13). ANS has a quantum yield of 0.004 in water solution, and 0.75—more than 100 times greater—when adsorbed upon BSA. Klotz (14) has shown that the adsorption of dyes on albumin may be characterized by the quantities n and K; n is the number of moles of dye absorbed by a mole of albumin, and K is the statistical dissociation constant of the dye-albumin complexes. These are related to the total protein concentration, P, dye concentration, D, and fraction of dye bound, x, by the equation:

\[
P/x D = \frac{1}{n} \left( 1 + \frac{K}{(1 - x)D} \right)
\]

Equation 3 gives,

\[
x = \frac{nP}{D_0 + K} = \frac{z + \frac{K}{D_0 + K} (x^2 + x^3 \ldots)}{D_0 + K}
\]

For values of x small in comparison with unity, x^2 and higher powers of x are negligible and the initial slope in the plot of x against P equals n/D_0 + K. If a concentration, D_0 >> K, is used, D_0 + K may be replaced by D_0 without appreciable error so that the initial slope equals n/D_0. On the other hand, having thus calculated n, a value of D_0 of order K may be used to determine K itself. This procedure has been used with intact BSA and with BSA submitted to the first rapid stage of pepsin digestion. x = F/F_0 where F_0 is the actual observed fluorescence efficiency when all the dye in solution has been adsorbed. F_0 was determined directly by adding excess BSA. F_0 remained constant for D_0 ≤ 10P, when P varied from 10^{-5} to 5 × 10^{-4} M. With initially digested BSA, F_0 was determined by a reciprocal plot of 1/F against 1/P. The value for adsorption on the digest was 6% higher than that for intact BSA. Intact BSA gave n = 5.02 and K = 3.2 × 10^{-6} m; initially digested BSA, n = 1.3 ± 0.3 and K = 5.6 × 10^{-6} m. The initial digestion appears to decrease both the number of molecules adsorbed and the affinity for each molecule. From Equation 4, if x ≪ 1,

\[
\frac{x_i}{x_f} = \frac{n_i}{n_f} \left( \frac{K_i + D_0}{K_i + D_0} \right)
\]

where the subscripts i and f refer to intact and fragmented BSA respectively. For D_0 >> K_f since K_f >> K_i,

\[
\frac{x_i}{x_f} = \frac{n_i}{n_f}
\]

For x ≪ 1 we also require P ≈ K_i in the last case.

Equations 5 and 6 show that it is possible to follow changes in n and K during digestion by using high (2.1 × 10^{-4} m) and low (7 × 10^{-7} m) ANS concentrations respectively. An experiment of this type is shown in Fig. 7. The figure shows that within the rapid phase of change of n it is possible to distinguish two distinct slopes, one corresponding to the first few minutes of digestion and another, larger, in the period immediately following. Clearly the possibility of investigating intermediate stages within the first phase of pepsin action exists, although we have not made use of it.

**Fluorescence Polarization of ANS-BSA Complexes**—The dependence of the polarization of the fluorescence, P, upon the rotational relaxation time, \( \tau_p \), and the lifetime of the excited state, \( \tau \), of the fluorescence is given by the equation:

\[
\frac{1}{P} = \frac{1}{P_0} + \frac{1}{\tau_p} + \frac{K_i}{P_0} \quad \text{if} \quad K_i \ll \frac{1}{\tau_p}
\]

The rotational relaxation time is a linear function of \( \eta/T \) (\( T \) is absolute temperature, and \( \eta \) is the viscosity coefficient of the solvent) so that a plot of 1/P against T/\( \eta \) should give a straight line with intercept 1/P_0 at T/\( \eta \) = 0. Using 1/P_0 thus calculated and the polarization, P, observed in water at 25°C we have

\[
\tau = \left[ \frac{1}{P} - \frac{1}{P_0} \right] \left[ \frac{1}{\tau_p} + \frac{K_i}{P_0^2} \right]^{-1} \quad \text{from which} \quad \tau \text{ may be calculated if } P_0 \text{ is known.}
\]

The reciprocal of the polarization of the fluorescence emitted by ANS adsorbates on bovine albumin as a function of T/\( \eta \) is shown in Fig. 8. The ratio of albumin to ANS molecules is 15:1. The observations were made at 25°, the viscosity of the solution being varied by the addition of sucrose, which even at high concentration has no influence upon the fluorescence efficiency of the adsorbates. With the use in Equation 7 of the value 118 nsec for the mean rotational relaxation time of albumin at 25°, \( \tau \) (the lifetime of the excited state of the fluorescence of the adsorbate) is 8.0 nsec. On the other hand, \( \tau \) may be calculated from the spectroscopic data of Fig. 9, which shows the absorption and fluorescence spectra of the adsorbates. The Perrin-Förster (15) equation for this purpose is

\[
\frac{1}{\tau_n} = 2.88 \times 10^4 n^2 \varepsilon_n \int E_\nu \, d\nu
\]

where \( \tau_n \) is the natural lifetime of the oscillator, n the refractive index of the medium surrounding the oscillator, \( \varepsilon_n \) the wave number of the absorption maximum, and E_\nu the molar absorption coefficient that is to be integrated over the absorption band. For the latter integration the Gaussian approximation (16)

\[
\int E_\nu \, d\nu = \sigma E_m \sqrt{\pi}
\]

has been used. E_m is the molar absorption coefficient at the
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G - 5

30 - 4

2 -

I I

IO 20 30 40 50 GO 70 ' Time (mins)

FIG. 7. Changes in relative number of molecules of ANS adsorbed upon BSA (n/n0) and ratio of apparent dissociation constants of adsorbates (K/Ki). Calculations were made from fluorescence efficiency measurements according to Equations 6 and 7 of text.

FIG. 8. Reciprocal of polarization (1/p) as a function of T/q for adsorbates of ANS on BSA at 25° in solutions of sucrose in water. The concentration of ANS was 1 × 10⁻⁴ M, and that of BSA was 10 mg per ml.

Maximum of absorption, and σ the half-width of the band in wave numbers. The latter is difficult to measure in the absorption spectrum, where it is masked partially by other electronic transition on the short wave side, but can be measured without ambiguity in the fluorescence spectrum as 2 of the total bandwidth. This is defined here (16) as the wave number interval determined by the two values of E = E0e⁻ⁿ. With n² = 2.2, δ0 = 26,450 cm⁻¹, σ = 1,900 cm⁻¹, and E∞ = 4.9 × 10⁻³ M cm⁻¹, τn = 11.8 nsec. The actual τ is related to τn and the quantum yield, q, by the relation τ = q·τn. With q = 0.75, τ = 8.5 nsec, in agreement with the value deduced from the polarization experiments.

Fluorescence Depolarization by Energy Transfer in ANS-BSA Complexes

If two or more molecules of ANS are adsorbed upon the same albumin molecule, with spatial orientations such that the emission oscillators are neither mutually parallel nor perpendicular, the emitted fluorescence is less polarized than that emitted by a complex containing a single molecule, provided the distance, r, between the adsorbed molecules is less than about ½ Ro, where Ro is the characteristic distance for the transfer of the excited state. If π is the probability that a single transfer from the excited to the unexcited molecule will take place during the lifetime of the excited state and 1 − π the probability of emission without transfer (17),

\[
\frac{\pi}{1 - \pi} = \left(\frac{R_0}{r}\right)^{16}
\]

(10)

Ro, the characteristic distance, is clearly that for which π = 1 − π = ½. In a theory due to Förster (17)

\[
R_0 = \left[\frac{1660}{\pi^2 \delta_0^2} \frac{\delta_0 + \delta_e}{2}\right]^{1/6} \times 10^{-6} \text{ cm}
\]

(11)

where δ0 is the maximum of absorption of the last band, δe the fluorescence maximum in wave numbers, and J0 the overlap integral. This, with the use of the Gaussian approximation (16), equals

\[
J_0 = E_0^e \left(\frac{1}{2 \pi} \frac{1}{\delta_0^2 + \delta_e^2}\right) \exp \left[-\frac{\left(\delta_0 - \delta_e\right)^2}{2 \delta_0^2 + \delta_e^2}\right]
\]

(12)

FIG. 9. Absorption and fluorescence spectra of ANS adsorbates of BSA. Fluorescence units adjusted to equalize maxima of absorption and emission, in order to give idea of extent of overlap.
The subscripts $a$ and $e$ refer to the absorption and fluorescence bands respectively. With the values obtained from Fig. 9 and $\tau = 8.0$ nsec from the polarization measurements, $J_f = 2.0 \times 10^8$ cm$^2$ ms$^{-1}$ and $R_a = 24$ Å.

Table V shows the polarization of the fluorescence of ANS adsorbed on serum albumin, as a function of the number, $n$, of moles of ANS adsorbed per mole of albumin present in solution. The decrease in polarization with the number of ANS moles adsorbed may be used to estimate the size of the adsorbing region in BSA. To be more precise, the polarization values can yield unambiguously only an upper limit for the average distance between the first two molecules adsorbed. With the additional assumption that in the population of adsorbing molecules all sites are equally likely to be occupied, the average distance between the first two adsorbed molecules becomes the average distance between all pairs of sites and is therefore directly related to the size of the adsorbing region.

Let $f_1$ represent the fraction of protein molecules carrying $n$ adsorbed ANS molecules ($0 \leq n \leq 5$), and $\varphi_n$ the contribution to the total fluorescent intensity by these molecules. The average number, $\bar{n}$, of molecules of ANS adsorbed per protein molecule is

$$\bar{n} = 2n \cdot f_n$$

and

$$\varphi_n = \frac{n \cdot f_n}{\bar{n}} f_n.$$  

If $\bar{n}$ is small compared to 1, we need not consider values of $n$ greater than 2, in which case, most sites being vacant,

$$f_2 \approx f_1^3$$

and therefore

$$\bar{n} = f_1 + 2f_2^2; \varphi_1 = \frac{2}{1 + \sqrt{1 + 8\bar{n}}}; \varphi_2 = \frac{\sqrt{1 + 8\bar{n}} - 1}{1 + \sqrt{1 + 8\bar{n}}}$$

so that for $\bar{n} \leq 1$

$$\varphi_1 = \frac{1}{1 + 2\bar{n}}; \varphi_2 = \frac{2\bar{n}}{1 + 2\bar{n}}.$$  

The quantities, $A = (1/p - 1/3)^{-1}$, called by Jablonski (18) the emission anisotropy, enjoy the property (3)

$$A = \sum_i A_i \varphi_i$$

where $A_i$ is the observed average value, $A_i$ the anisotropy value for the $i$th component when alone in solution, and $\varphi_i$ its fractional contribution to the total fluorescent intensity of the system. For the case of our two components

$$A = A_1 \varphi_1 + A_2 \varphi_2$$

which together with Equation 16 gives

$$A (1 + 2\bar{n}) = A_1 + 2\bar{n} A_2.$$  

Fig. 10 shows the plot of $A (1 + 2\bar{n})$ against $2\bar{n}$. For $\bar{n} \ll 1$, the expected linear relation is fulfilled. The intercept, 0.340, is the emission anisotropy for a pure $n = 1$ population; the limiting slope, 0.260, is the emission anisotropy for a population with $n = 2$. For this last one, the theoretical treatment of Förster (17) gives as the contributions $\psi_1$ and $\psi_2$ of the originally photo-

<table>
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<th>Polarization</th>
<th>ANS adsorbed</th>
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<tr>
<td>0.303</td>
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<tr>
<td>0.218</td>
<td>5.0</td>
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</table>

![Fig. 10. Plot of emission anisotropy, $A = (1/p - 1/3)^{-1}$ against $2\bar{n}$ (8 = average number of moles of ANS adsorbed per mole of BSA). The intercept and the slope are, respectively, the values of $A$ for homogeneous populations with 1 and 2 moles of ANS adsorbed upon 1 BSA molecule.](http://www.jbc.org/)

Fig. 10 shows the plot of $A (1 + 2\bar{n})$ against $2\bar{n}$. For $\bar{n} \ll 1$, the expected linear relation is fulfilled. The intercept, 0.340, is the emission anisotropy for a pure $n = 1$ population; the limiting slope, 0.260, is the emission anisotropy for a population with $n = 2$. For this last one, the theoretical treatment of Förster (17) gives as the contributions $\psi_1$ and $\psi_2$ of the originally photo-
the value of $\pi$ determined is a minimal value. For random transfer, $A_1 = 0.04 A$.

For random transfer, $A_2 = 0.20 A$, $A_1 = 0.340$, and $A_2 = 0.04$, gives $\pi = 0.33$, and this value introduced with $R_s = 24 A$ in Equation 10 gives $\pi = 27 A$.

If the two molecules first adsorbed were distributed at random over the surface of a sphere of 50 A diameter (corresponding to an anhydrous molecular weight of 88,000), the expected value of $\pi$ would be 0.18 (19). It must be concluded that the binding rules are adsorbed upon a restricted region of the BSA surface, an anhydrous molecular weight of 68,000), the expected value from a medium of low polarizability (22, 23). If the dye molecules are adsorbed upon a restricted region of the BSA surface, as must indeed be the case if the adsorption takes place upon the surface forming the common boundaries between the globular fragments, the adsorption of several fluorescent dye molecules should in principle result in depolarization of the fluorescence by transfer of the excited state. Such effect is clearly observable when ANS is adsorbed upon BSA. With some assumptions concerning the random character of the adsorbing process, the average distance between the two first adsorbed ANS molecules has been found to be 27 A, which is to be compared with the value of 33 A expected as the average distance of separation for random distribution of pairs over the surface of a spherical molecule of the same anhydrous volume as serum albumin.

A model of the albumin molecule consisting of compact fragments and flexible links between them has already been proposed by Foster (24) on the basis of the behavior of albumin at acid pH, and on the interaction of albumin with detergents. The pepsin digestion experiments discussed, together with the isolation of the fragments in high yield described in the following paper, are in complete agreement with this model.

**SUMMARY**

1. The fluorescence polarization of conjugates of bovine serum albumin with 1-dimethylaminonaphthalene-5-sulfonate is decreased by 0.001 of its weight of pepsin at pH 3.0 shows a phase of rapid decrease followed by one of much slower change.

2. After the rapid phase, the rotational relaxation time becomes independent of the pH of the solution; i.e., the pepsin treatment abolishes the well-known acid effect.

3. The sedimentation constant of intact bovine serum albumin undergoes a similar biphasic change upon pepsin digestion.

4. A rapidly reversible concentration-dependent association of the fragments of pepsin digestion is observed as an increase in sedimentation constant and fluorescence polarization with total protein concentration.

5. Five molecules of the polycyclic aromatic anion, 1-anilinonaphthalene-8-sulfonate are absorbed at pH 7 by 1 molecule of serum albumin with a statistical binding constant, $K = 3.2 \times 10^{-5}$. The binding power of bovine serum albumin towards this anion shows a biphasic decrease during pepsin digestion with respect to both $K$ and the maximal number, $n$, of molecules adsorbed. After the initial phase, $n = 1.3 \pm 0.3$ and $K = 5.6 \times 10^{-5}$.

6. The polarization of the fluorescence of 1-anilinonaphthalene-8-sulfonic acid adsorbed upon bovine serum albumin decreases monotonically as the number of moles of the acid adsorbed per mole of albumin increases, as a result of migration of the excited state.

7. A calculation assuming random orientation and equal binding power of all binding sites in the bovine serum albumin molecule gives 27 A as the distance between a pair of such sites.

8. The preceding observations may be explained on the as-
sumption that the action of pepsin liberates a small number of compact globular fragments by breaking up peptide bonds in the flexible links between them, and that adsorption of L-anilino-
naphthalene-8-sulfonic acid takes place upon the binding surfaces common to two or more of the compact fragments in the intact molecule.

Acknowledgments—The possibility of using proteolysis of acid solutions of BSA as a test of the proposed model of this protein was suggested to us by Dr. Parker Small. It is a pleasure to acknowledge here our debt. We are also indebted to Dr. Finn Wold for his interest and help at all times.

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