A Study of the Adsorption of Dyes on Bovine Serum Albumin by the Method of Polarization of Fluorescence

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The ability of serum to bind small molecules was brought into prominence by the work of de Haan (1922) on the excretion of vital dyestuffs by the kidney. Grollman (1925) later showed by ultra-filtration that serum albumin possesses the property not possessed by ovalbumin, serum globulin, or gelatin, of adsorbing phenol red at alkaline pH. He showed that all the proteins studied bound the dye when the dye and the protein were of opposite electrical charge, but that serum albumin could also bind the negatively charged dye when it was itself negatively charged, adsorption decreasing rapidly, however, above pH 8. Bennhold (1932) showed that a variety of dyes and substances of pharmacological value was carried by the albumin fraction of serum and proposed for the protein a transporting function in the animal body. Recent studies on the binding by the serum albumin fraction (Smith & Smith, 1938; Rawson, 1943; Chow & McKee, 1945) and especially the detailed studies of Murray Luck and his co-workers on the stabilization of serum albumin (Boyer, Lum, Ballou, Luck & Rice, 1946) and the intensive physicochemical studies of Klotz (1949) have gone far to elucidate both the conditions under which adsorption takes place and the practical importance of the phenomenon (Edsall, 1947). Meanwhile, Scatchard, Scheinberg & Armstrong (1950) and Edsall, Edelhoch, Lontie & Morrison (1950) have shown that serum albumin also binds a number of negatively charged inorganic ions.

The present paper is concerned with a study similar in principle to those previously carried out, of the binding of certain fluorescent dyes to bovine serum albumin. The method used was that developed by Dr G. Weber in this department depending on a measurement of the polarization of the light emitted by excited fluorescent molecules (Weber, 1952).

THEORETICAL

The polarization of the fluorescent light from a dye molecule depends on the relaxation time of rotary Brownian movement of the dye in a given environment and on the time which the dye molecule takes to emit its light following an excitation. If the dye

molecules are able to rotate extensively between excitation and emission, the fluorescent light has little overall polarization while if the molecules rotate very little in this time the emitting oscillators will remain appreciably orientated and will produce an overall polarization of the fluorescent light (Perrin, 1936). For the majority of dyes in aqueous solution the relaxation times of the molecules are small compared with the time taken to emit the fluorescence, and so the polarization of the fluorescence is small (usually less than 1%). The relaxation time may be increased by placing the dye in a viscous medium such as glycerol (Weigert, 1920) or, as Weber has shown, by attaching the dye to a macromolecule such as a protein, which rotates more slowly in aqueous solution than does the free dye. The result of importance in the present study is that the fluorescence of the dye adsorbed on the protein is partially polarized, while the fluorescence of the free dye is usually unpolarized. Therefore, in any mixture of a fluorescent dye with a protein, where a part of the dye is bound to the protein and the remainder is free, the polarization P observed will be less than that for complete binding of the dye P_b depending on the relative contribution which the free and the bound dye make to the total fluorescent intensity. One can say that the polarized fluorescence of the bound dye is diluted by the unpolarized fluorescence of the free dye. A knowledge of the relative intensities of fluorescence of the dye, free and bound, and of the measured polarization of the dye-protein mixture enables the amount of dye bound to be easily calculated as follows. (For derivations of these formulae see Appendix.)

Assuming that the fluorescence of the free dye in solution is unpolarized, the ratio of P to P_b will vary between 0 and 1 as the degree of adsorption of the dye on to the protein is varied. Calling this ratio $\bar{p}$ and supposing the intensity of the fluorescence of the dye-protein system relative to that of the dye alone at the same concentration and pH is R it is shown that the fraction of dye bound (x) is

$$x = \bar{p}R - R + 1. \quad (1)$$

If the dye does not change its fluorescent intensity on adsorption (i.e. $R = 1$) the simple formula

$$x = \bar{p} \quad (1a)$$

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will hold. On the other hand, if the free dye in aqueous solution possesses a measurable polarization a second modification of Eqn. 1 is needed, namely that, where $\Pi = P_f/P_B$,

$$(1 - \Pi)x = \bar{p}R - R + 1 - \Pi, \quad (1b)$$

and P_f is the polarization of the free dye in solution.

Only one case was found in this work for which the simple formula (1a) could be used (see below); most of the dyes studied showed either a marked increase or a decrease in fluorescence when bound to bovine serum albumin. The significance of these changes in fluorescence will be discussed later, but it is enough to note here that if a marked increase in fluorescent yield occurs on adsorption (as in the case of the sulphonic acid derivatives of 1-naphthylamine), formula (1) ceased to be of practical use since the observed polarization is little diluted by the relatively poor fluorescence of the free dye. In these cases a formula

$$x = \frac{R - 1}{R_{\max} - 1} \quad (2)$$

has been used, in spite of the fact that this formula does not distinguish between a change in the fraction bound (x) and a redistribution of the already bound molecules among adsorption sites which affect the fluorescence to different degrees. A limitation of this sort is implicit in any indirect method such as this, especially where only one experimental variable is used, and may equally enter into the spectrophotometric technique (Klotz, 1946b). Since formula (1) includes two experimentally determined variables it is less likely to suffer from limitations of this sort and the formula has been used to detect one case (eosin) where the sites are of different kinds.

In order to determine the number of adsorption sites on the albumin and their affinity for the dye the equation derived by Klotz (1946a) was used, namely,

$$\frac{S}{Dx} = \frac{1}{n} + \frac{K}{nD(1-x)}. \quad (3)$$

The known quantities in this equation are S and D , the total protein and dye concentrations respectively, and x , the fraction of dye bound, calculated as described above. The unknown quantities K , the equilibrium constant of dissociation of the eye-protein complex, and n the number of adsorbing sites, may easily be derived from the experimental curves, since n is the reciprocal of the intercept at the axis and K/n the slope of the graph. A greater slope means a more dissociable complex. When the dye concentration is small compared with the protein concentration the simple formula

$$nS \frac{(1-x)}{x} = K \quad (3a)$$

will apply. This is a useful formula since, even if the adsorbing sites differ in combining power, the form of the equation remains the same.

EXPERIMENTAL

Dyes

The dyes used were chosen for their availability and possible experimental value. They can be classified chemically into three classes: (i) sulphonic acid derivatives of 1- and 2-naphthylamine containing one, two or three sulphonic acid groupings; (ii) derivatives of fluorescein and rhodamine with a xanthidrol ring system; (iii) derivatives of acridine. Where necessary these dyes were recrystallized or converted to crystalline derivatives and recovered. The purity of the dyes was examined by chromatography on paper. The term 'dye' has been used for these fluorescent compounds to simplify the nomenclature, although in some cases the absorption maximum of the compounds lies in the near ultraviolet region of the spectrum.

Serum albumin

The protein used was the Armour crystalline bovine serum albumin (batch no. 10522). It was found by comparison with the Armour fraction 5 albumin (batch no. 12 198) that the crystallization had not appreciably altered the adsorbing properties of the protein, but had removed some yellow material whose absorption of the exciting light might complicate the fluorimetric studies. The absorption at the excitation wavelength of 3600 Å. was not great enough in the crystalline product to require any special corrections. The fluorescence of the crystalline protein was also unimportant with the yellow filters (Corning 338, 351, etc.) used for examining fluorescence. The protein was made up in 1% solution, dialysed for 24 hr. at 0° and filtered through Whatman no. 50 filter paper to give a clear solution. The protein concentration was measured in a Zeiss interferometer using the diffusate in the control compartment. The interferometer was calibrated using Kjeldahl nitrogen values for ovalbumin solutions.

Measurement of fluorescence

The protein and dye were mixed and a suitable buffer added. Experiments were made both at constant dye concentration and at constant protein concentration. When a series was carried out at constant dye concentration, only one comparison tube for free dye was required. When the dye concentration was varied, a comparison tube was made up for each dye concentration used. Observations were made in each case on the fluorescent intensity of the dye-protein mixture, as compared with the fluorescence of free dye; and on the polarization of the fluorescence of the dye-protein mixture. In cases where interest was found in the absorption spectrum of the mixture, this was measured in the Beckman spectrophotometer from 6000 Å. down to 3100 Å.

The limiting polarization P_B was measured by so increasing the protein concentration that no further increase in polarization resulted from addition of more protein. This condition was obtained with the dyes at certain pH values where binding was especially strong. The work of Weber (1952) suggests that the same value of P_B would be valid at other pH values.

Since polarization measurements are most easily made visually, the intensity measurements were also made visually, the same filters for excitation and observation being used in both cases. Any changes in colour in the

fluorescence during adsorption would affect intensity measurements made with a photocell in an arbitrary way with respect to the eye and make the use of Eqn. 1 more difficult. Fluorescence of wavelength short enough to be reabsorbed by the dye was excluded from observation by choice of filters. The visual fluorimeter was made by placing a Pulfrich photometer headpiece vertically at the end of an optical bench and splitting the beam of the usual Siemens mercury lamp, placed on the bench, by means of a modified Pulfrich beam-splitter. The cells containing the fluorescent solutions, size $4 \times 2 \times 1$ cm. were filled completely with the solutions and covered with pieces of optical glass. They were

tion of the exciting light (Weber, 1952). In the measurement of polarizations the exciting light was polarized by a Nicol prism in a plane perpendicular to the axis of observation. In the fluorimeter, on the other hand, the exciting light was unpolarized. To make the two sets of measurements comparable the following formula (derived in the Appendix) was used where I' is the observed and I is the corrected intensity:

$$I = \frac{I'}{1 - \frac{1}{2}p}$$

For the theoretical maximum of the polarization of 0.50 this correction is 33%.

Table 1. *Combination of fluorescein in borate buffer at pH 9.1*

(Fluorescein concentration = 0.13×10^{-5} mol./l.)

Protein concn. ($\times 10^5$ mol./l.) <i>S</i>	Polarization ($\times 100$) $100p$	Relative intensity <i>R</i>	$\bar{p}R$	Fraction of dye bound (<i>x</i>)	Equilibrium constant ($\times 10^5$) $K = nS(1-x)/x^*$
1.46	7.6	0.61	0.13	0.52	1.35 <i>n</i>
2.92	12.0	0.51	0.17	0.66	1.50 <i>n</i>
5.84	17.0	0.37	0.17	0.80	1.45 <i>n</i>

* Where *n* is the number of binding sites.

illuminated on their largest faces and observed through the top. The fluorescent intensities of a series under test were always compared with the brightest fluorescence of the series whether the free dye or the dye combined with the protein.

A simple correction was made to the intensity measurements, since the intensity observed from a solution producing polarized fluorescence is dependent on the polariza-

RESULTS

Table 1 gives some typical results of the combination of the bivalent negative ion of fluorescein with serum albumin in borate buffer at pH 9. The fluorescein concentration was small compared with the protein concentration and so Eqn. 3*a* applies.

The result of plotting according to Eqn. 3 is a straight line passing through the origin with slope determined by the equilibrium constant.

Table 2 illustrates a second simple case. This is that of the tervalent negative ion of the 2-naphthylamine-3:6:8-trisulphonic acid in unbuffered salt-free protein solution at pH 5.4. In this case there is strong binding to the protein so that over a range of concentration of both protein and dye two molecules of the dye are bound by one molecule of albumin (molecular weight 70 000). Extrapolation from Table 1 shows that fluorescein is only 0.26 times as fluorescent on the protein as in the solution. The fluorescence of the 2-naphthylamine-3:6:8-trisulphonic acid, on the other hand, is but little altered by adsorption.

Table 2. Combination of 2-naphthylamine-3:6:8-trisulphonic acid at the isoelectric point

Protein concn. $\times 10^5$ mol./l.	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
(a) Dye concentration $= 5 \times 10^{-5}$ mol./l.		
0.60	0.26	2.15
1.21	0.52	2.15
2.42	0.92	1.89
(b) Protein concentration $= 1.21 \times 10^{-5}$ mol./l.		
Dye concn. ($\times 10^5$ mol./l.)	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
5.0	0.50	2.06
10.0	0.258	2.13
20.0	0.132	2.18

Table 3 gives data for the monovalent negative ion of 1-naphthylamine-8-sulphonic acid in phosphate buffer, pH 6.9. In this case the fluorescence of the dye is much increased on the protein (about

Table 3. Combination of 1-naphthylamine-8-sulphonic acid at pH 6.9

(Sulphonic acid concentration 4.15×10^{-5} mol./l.)

Protein concn. ($\times 10^5$ mol./l.)	Relative intensity ($\div 20$)*	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
0.00	0.05	0.00	—
0.725	0.37	0.34	1.95
1.45	0.62	0.60	1.72
2.90	0.84	0.84	1.19
7.25	1.00	1.00	—

* I.e. relative to the same concentration of free dye, allowing for the increase of fluorescence on adsorption.

20-fold), and the polarization rises to a maximum before the limit is reached in the increase of fluorescence. Using Eqn. 2 it appears that about 2 molecules of this substance are bound to the protein in the concentration range studied.

Effect of pH

As the pH in the surrounding solution is decreased and the charge of protein becomes more positive it would be expected that there would be an increase in the amount of a negative dye bound to

Fig. 1. Adsorption isotherms of 2-naphthylamine-3:6:8-trisulphonic acid.

Fig. 2. Adsorption isotherms of fluorescein.

the protein. This is borne out by the following data for 2-naphthylamine-3:6:8-trisulphonic acid (Fig. 1), fluorescein (Fig. 2) and 1-naphthylamine-3:8-disulphonic acid (Fig. 3). It should be noted that fluorescein contains two titratable groups between pH 8 and 4, while the 1-naphthylamine-3:8-disulphonic acid contains one with pK 3.9. These reduce the negative charge on the dye at acid pH relative to alkaline pH.

Figs. 4 and 5 summarize the results obtained by plotting the polarizations obtained at an arbitrary protein concentration against the pH. Fig. 4 is for positive ions (basic dyes) and Fig. 5 is for negative ions (acidic dyes). The results illustrate the familiar observation that an opposite charge on the protein favours the combination of a dye, but there is also

lipid-soluble) is more strongly adsorbed than the positive ion, even though the average charge on the protein at these pH's would be expected to favour combination with the positive ion.

This is confirmed by the result of adding serum albumin to *o*-5-acridylbenzoic acid at pH 6 where the protein is slightly negative and the dye mostly in the zwitterion form with a negatively charged

Fig. 3. Adsorption isotherms of 1-naphthylamine-3:8-disulphonic acid.

Fig. 4.

Fig. 5.

Fig. 4. Effect of pH on the adsorption of positive dyes (a) mepacrine, (b) cresyl fast violet, (c) 5-aminoacridine, (d) acridylbenzoic acid methyl ester.

Fig. 5. Effect of pH on the adsorption of negative dyes (a) fluorescein, (b) 1-naphthylamine-3:8-disulphonic acid, (c) 2-naphthylamine-3:6:8-trisulphonic acid, (d) eosin.

noticeable the special characteristic of serum albumin. This is apparent in both graphs. In Fig. 5 it is seen that negative ions, even tervalent ions, continue to combine with the protein on the alkaline side of the isoelectric point (pH 5.4). In Fig. 4, though the basic dyes combine in all cases with the protein, there are marked changes in extent of combination at the pK's of certain groups in the dyes. These changes lead to the conclusion that the neutral form of the dye (that form which is more

Fig. 6. Effect of pH on the absorption spectrum of *o*-5-acridylbenzoic acid.

Fig. 7. Effect of the addition of serum albumin on the absorption spectrum of acridylbenzoic acid at pH 6.0.

carboxyl group and a positively charged heterocyclic ring. The serum albumin has the same effect on the dye as does a change of pH toward the alkaline side (Figs. 6 and 7). Addition of serum albumin to the dye at pH 9 when the heterocyclic nitrogen is uncharged has no effect on the spectrum. This 'reversed protein error' can be due to forces like those which produce the other effects discussed here. Fig. 8 shows adsorption isotherms for this dye.

That the neutral and ionic forms of the dyes differ markedly in the P_b values is made unlikely by experiments on the polarization of the fluorescence of the two forms in glycerol-water solutions of various viscosities. The conclusion follows that

while the presence of a charged group on the molecule is not a necessary condition for adsorption to occur, a positive group in contrast with a negative group has a strong inhibitory influence on the combination. This influence extends as far as pH 9.3, the pK of the ring nitrogen in 5-aminoacridine (Albert & Goldacre, 1943) when the protein has a net charge of about -30 (Tanford, 1950).

Fig. 8. Adsorption isotherms of acridylbenzoic acid.

Effect of modifications in structure of the dye

Some results of interest to the general theory of adsorption are obtained when the effect of minor alterations in the structure of the dye on its adsorption properties is studied. Fig. 9 shows results with fluorescein as compared with eosin in neutral phosphate buffer. Eosin is a case where the intensity does not change linearly with the calculated fraction bound (x) but first decreases and then increases as the protein concentration is increased. This unusual effect can be explained if only a few of the total binding sites yield an appreciably fluorescent adsorption complex and if it is assumed that these are the most actively combining sites. While the dye is in excess of the total sites, both the sites of low and of high fluorescent yield will be occupied. If an excess of sites is present, however, the sites of higher fluorescent yield will be favoured and the fluorescent intensity will increase again. There is therefore a minimum of fluorescence depending on the ratio of dye to protein. Using the formula 1 b the amount bound is found to increase continuously over the range of protein concentration.

The naphthylaminesulphonic acids tried, namely the 1-naphthylamine-5- and 8-monosulphonic acids, the 3:8-disulphonic acid and the 3:6:8- and 4:6:8-trisulphonic acids and the 2-naphthylamine-3:6:8-sulphonic acid, showed binding of about the same extent at neutral pH. Methylation of the amino group increases the extent of combination of the 1-naphthylamine-5-sulphonic acid with serum albumin.

It can also be shown that fluorescein methyl ester and *o*-5-acridyl-benzoic acid methyl ester are adsorbed to about the same extent as the free acids. Sulphofluorescein is adsorbed similarly to fluorescein, and succinylfluorescein, with a short side chain instead of a ring, less than fluorescein.

Fig. 9. Adsorption isotherms of eosin and fluorescein in 0.01 M-phosphate pH 6.9.

Competition experiments

As it has so far been described, the method of the polarization of fluorescence is limited to studying the combination of fluorescent substances only. A knowledge of the ability of various substances to compete with suitable test dyes leads, however, to an extension of the method in a more indirect form (Klotz, 1949). There are two easy ways of carrying out competition experiments:

(i) With a given quantity of serum albumin and of test dye the polarization P_1 is recorded and, in addition, the polarization P_2 (always less than P_1) which results when a known further amount of the same dye is added. If, instead of this further addition, an equimolar amount of a non-fluorescent competitor is added and a polarization P_3 results we have either

$P_3 = P_1$	The substance shows no competition
$P_3 = P_2$	Competes as strongly as the test substance
$P_1 > P_3 > P_2$	Competes less than the test substance
$P_3 < P_2$	Competes more than the test substance

This is illustrated by Table 4, where various derivatives of naphthalene are used to compete with the 2-naphthylamine-3:6:8-sulphonic acid. The sulphonic groups, but not the amino groups, seem necessary for competition. As the 1- and 2-naphthylamines themselves have no effect on the polarization, it seems likely that they do not combine at the site of combination of the test substance at the concentration used.

(ii) For substances whose combination with protein is fairly weak, competition can be studied by adding the competitor in great excess relative to the protein and noting the decrease in the polarization.

Table 4. *Competition of related compounds with 2-naphthylamine-3:6:8-trisulphonic acid* (pH 6.7)

Competitor	Amount of test substance (ml.)	Amount of competitor (ml. equimolar solution)	Polarization
None	1.0	—	0.060 P_2
None	0.2	—	0.116 P_1
1-Naphthylamine	0.2	0.8	0.115 P_3
2-Naphthylamine	0.2	0.8	0.117 P_3
Naphthalene-1-sulphonic acid	0.2	0.8	0.065 P_3
Naphthalene-2-sulphonic acid	0.2	0.8	0.052 P_3
Naphthalene-3:6:8-trisulphonic acid	0.2	0.8	0.035 P_3
N-Acetyl-1-naphthylamine-8-sulphonic acid	0.2	0.8	0.071 P_3

The present experiments have been confined to comparing the relative affinities of related simple substances as revealed by this method, although an absolute derivation should be possible.

Table 5 gives data for the ability of various ions to compete at the same molar concentrations, again using the 2-naphthylamine-3:6:8-trisulphonic acid as test substance. The outstanding trend in this table is that whereas alteration of the cation has little or no effect on the extent of the combination the ability of different anions to compete is markedly different. This agrees with the results found both in this study and by other workers that preferential combination occurs with the negative ion of an ionic compound in solution.

The competition with the series of univalent anions $F^- < Cl^- < Br^- < NO_3^- < I^-$ lies in their order in the so-called Hofmeister series relating to gelatin (Loeb, 1924) and this shows that the simple assumptions of the Debye-Hückel theory cannot in this case be sufficient to explain the effect of these salts. The effect of bromine atoms (see results with eosin above) and of nitro groups (Teresi & Luck, 1948) in organic molecules seem to be paralleled by the strong binding of the nitrate and bromide ions.

Table 6 gives a similar set of figures for some organic anions and one cation. The differences observed between the different compounds in this

Table 5. *Competition of some ions with 2-naphthylamine-3:6:8-trisulphonic acid* (pH 6.7)

Competitor (M/15):	Polarization
None	0.202
NaCl	0.100
KCl	0.100
Na_2SO_4	0.015
K_2SO_4	0.015
Sodium acetate	0.130
Sodium succinate	0.015
Sodium benzoate	0.000
NH_4Cl	0.100
CsCl	0.101
NaF	0.181
KCl	0.098
KBr	0.050
KI	0.000
NaN_3	0.045
$NaNO_3$	0.015
Competitor (M/30):	
K_2SO_4	0.045
$(NH_4)_2SO_4$	0.047
Li_2SO_4	0.048
$MgSO_4$	0.070

table are of importance for the theory of adsorption discussed below.

The 2-naphthylamine-3:6:8-trisulphonic acid was used in these studies because its fluorescence intensity is little changed on adsorption and its

Table 6. *Competition of organic ions with 2-naphthylamine-3:6:8-trisulphonic acid* (pH 6.7)

Competitor	Competitor concn. ($M \times 10^{-2}$)				
	0	1	1.5	3.5	6
	Polarization				
Sodium galacturonate	0.205	0.205	0.205	0.205	0.205
Sodium phenylacetate	0.205	0.162	0.102	0.000	0
Sodium cyclohexylacetate	0.205	0.071	0	0	0
Sodium caprylate	0.205	0	0	0	0
Octylamine hydrochloride	0.205	0.205	0.205	0.148	0.095*
	Competitor concn. ($M \times 10^{-3}$)				
	0	1	1.5	2.5	
	Polarization				
Sodium caprylate	0.208	0.140	0.067	0	

* Attributable to the chloride ion alone.

polarization has, therefore, a linear relationship to the fraction which is bound. The next section considers more generally the changes in fluorescent properties and absorption spectra of the dyes.

Change in fluorescence and absorption spectra on adsorption

In the previous sections the change in fluorescent intensity on adsorption has been mentioned as an important factor to be considered in applying the method of the polarization of fluorescence to a study of adsorption isotherms. During adsorption there are also changes in the absorption spectra of the dyes. The changes may be summarized conveniently according to the chemical types of the dye molecules.

(i) *Sulphonic acid derivatives of 1- and 2-naphthylamine*. The fluorescence of the 2-naphthylamine-3:6:8-trisulphonic acid is bright sky blue and is slightly quenched when the dye is on the protein without a change in colour. The fluorescences of the 1-naphthylamine derivatives are greenish yellow in aqueous solution and this is also the case with the dimethylaminonaphthalene derivative. The fluorescence of all these compounds is increased when they are adsorbed on the protein and in most cases it is changed to the bright blue of the 2-naphthylamine compound described above. This change is most marked with the relatively weak fluorescence of the 1-naphthylamine-8-sulphonic acid where a 20-fold increase in brightness occurs. With the stronger fluorescence of the others in this series, increases of two or fourfold are observed. The change may be due to an increase in the dipole moment of the amino group on adsorption, as it is known that 2-naphthylamine possesses a higher dipole moment than 1-naphthylamine (Bergmann & Weizmann, 1936). Shifts to the red of 80 Å. were observed in the ultraviolet spectra of the 1-naphthylamine compounds when adsorbed.

(ii) *Xanthidrol derivatives*. The fluoresceins and rhodamines tried were all quenched on the protein. For fluorescein itself the intensity was 0.26 of that of the free dye. In addition, characteristic shifts in the absorption spectrum towards the red occurred. This effect was most marked with the methyl ester of fluorescein where the sharp absorption bands of the free and of the adsorbed dyes are clearly separated at the maxima, while the shape and height of the curves are hardly altered (Fig. 10). The colours of solutions of the adsorbed methyl ester are bright pink while that of the free dye at alkaline pH is orange yellow. This is not an effect of pH, as the absorption of the different ionic forms of fluorescein moves progressively towards the blue with increased acidity. In the case of eosin the adsorbed dye has a moderate adsorption at the green line of mercury, namely, 5460 Å. while the free dye absorbs little at

this wavelength. The tubes which contain adsorbed material can therefore be distinguished easily as they are brightly fluorescent in this light, whereas tubes with the free dye alone are by comparison practically non-fluorescent.

(iii) *Acridine derivatives*. Considerable variation was found in the behaviour of the fluorescences of the acridines when adsorbed. The fluorescence of o-5-acridylbenzoic acid was quenched to one-tenth of its value in solution without change in its absorption spectrum. The fluorescence of mepacrine increased slightly.

Fig. 10. Absorption spectrum of fluorescein methyl ester at pH 9 (a) free dye, (b) dye adsorbed on serum albumin or cetyltrimethylammonium bromide.

The relation of the changes in fluorescences and absorption spectra to the state of the environment of the dyes

Since the change in the fluorescences and the absorption spectra of the dyes is likely to be characteristic of some new electronic state due to an environment at the site of adsorption different from that in water, it was considered likely that a simulation of these changes in simple physico-chemical systems would lead to an understanding of the nature of the adsorbing region on the protein. A suitable series of dyes was chosen to test this, namely, eosin, fluorescein methyl ester, 1-dimethylaminonaphthalene-5-sulphonic acid, 1-naphthylamine-8-sulphonic acid and o-5-acridylbenzoic acid. By trial it was found that the cationic detergent, cetyltrimethylammonium bromide (CTAB), gave reactions with these dyes very similar to those given by bovine serum albumin. That adsorption by the detergent micelles had occurred was demonstrated by showing that the detergent in low concentration increased the polarization of eosin excited with the

green line of the mercury lamp and of fluorescein excited with the blue lines. Klotz (1947), using the azo-dye methyl orange, has shown a similar parallel between the effect of protein and of CTAB on the absorption spectrum. The ring structure of the dye which acts as the oscillator (Benel, Kastler & Rousset, 1940) is likely to be in the micelle rather than adsorbed on its surface (Hartley, 1937), and hence in a substantially organic environment free from water dipoles.

An important property of the interior of the micelle is its low dielectric constant, and in a single-phase system the dielectric constant of the solutions of the dyes was lowered by adding dioxan (Akerlof & Oliver, 1936) which had been purified by the

Fig. 11. Effect of dioxan concentration on the absorption spectrum of fluorescein methyl ester (from right to left, increasing dioxan concentrations from 0 to 90 %.)

Fig. 11a. Shift in the wavelength of the absorption maximum with increasing dielectric constant of the dioxan-water mixtures.

method of Oxford (1934). The dyes behaved in a way similar to that observed when they were adsorbed by serum albumin and CTAB, the most striking similarities being shown by fluorescein methyl ester, 1-naphthylamine-8-sulphonic acid and acridylbenzoic acid (Figs. 10–13). The effect of solvents of low dielectric constant in imitating the effect of serum albumin is such that we could almost without exception predict how a given dye would change its fluorescence and spectrum from its behaviour in dioxan solution. Evidence that the dielectric constant of the medium is the determining factor in the changes in spectrum of the xanthidrol compounds may also be obtained from the results of Vaillant (1927) for erythrosin, though this author does not interpret his results in this way. He found that the largest shifts to the red in the spectrum of erythrosin were in benzene and 'essence', both sol-

vents of non-polar nature. Glycerol, concentrated sugar solution and ethanol gave smaller shifts to the red with respect to water, although the refractive indices and hence the volume-polarizabilities of these liquids was as high or higher than the benzene and 'essence'. A similar view is admitted by Seshan (1936).

Fig. 12. Effect of dioxan on the absorption spectrum and fluorescent intensity of 1-naphthylamine-8-sulphonic acid. (a) spectrum of dye in aqueous solution, (b) spectrum of dye adsorbed on serum albumin or in 90 % dioxan solution.

It may be concluded that the behaviour of the fluorescent dyes when adsorbed on to serum albumin identifies the site of adsorption with material of low dielectric constant. The use of a series of these dyes should enable other systems of amphipathic behaviour to be easily distinguished as has already been stated (Sheppard & Geddes, 1945).

Relation to the work of Dr G. Weber with fluorescent groups coupled by chemical reaction with proteins

Of the dyes used in this paper, two, namely the 2-naphthylamine-3:6:8-trisulphonic acid and the 1-dimethylaminonaphthalene-5-sulphonic acid, have

been used by Dr G. Weber as compounds suitable for coupling with proteins by covalent bonding. It is possible to carry out studies using serum albumin similar to those of Weber (1952) by ensuring that all the dye is combined with protein and plotting a curve of $1/P_B$ against T/η , where T is the absolute

eosin excited by Hg 5460 Å. where an extrapolation of $1/P_B$ to $T/\eta = 0$ gives the accepted value for eosin in very viscous solution. The colour of the fluorescence of the serum albumin-sulphonamide conjugate is greenish blue like the colour of fluorescence of the simple sulphonamides in 90% dioxan. Digestion of the albumin conjugate with Armour crystalline pepsin results in the fluorescence becoming orange like that of the sulphonamides in water. Even with native proteins the greenish-blue fluorescence of albumin conjugates is not universal (Weber, 1952) and the yellowing of the fluorescence of the sulphonamides as the polarity of the medium is increased may provide a convenient yardstick against which the polarity of the protein surface in the neighbourhood of the primary amino groups may be estimated.

Fig. 13. Effect of dioxan on the absorption spectrum and fluorescent intensity of *o*-5-acridylbenzoic acid. (a) spectrum of dye in aqueous solution, (b) spectrum of dye in 90% dioxan.

temperature and η the viscosity. The intercept of this line with the ordinate gives the value of $1/P_B$ in the absence of Brownian movement, and the slope is determined by the lifetime of the excited state and by the size of the protein. In either case the value of $1/P_B$ was found to be the same at $T/\eta = 0$ for the coupled as for the adsorbed dye. The slope of the graph for the adsorbed 1-dimethylaminonaphthalene-5-sulphonic acid was found to be twice that for the coupled sulphonamide conjugate (Fig. 14). Since Weber (unpublished observations) has shown that the lifetime of excited state of the acid in glycerol is about twice that of the derived sulphonamide, these results show that the adsorbed dye has a fixity in the albumin surface comparable with that of the coupled dye. A similar result is obtained for

Fig. 14. Plot of polarization at different temperatures according to the Perrin equation for the 1-dimethylaminonaphthalene-5-sulphonic acid attached to serum albumin (a) covalently coupled (Weber's data), (b) adsorbed.

DISCUSSION

The evidence presented here and that obtained by other workers leads to the view that there are several kinds of physicochemical mechanism operating in the process of adsorption on to serum albumin.

It is clear that the charge on the molecule is of importance, negative ions being adsorbed in preference to positive ions at the sites of adsorption. Superimposed on this is a variation in binding depending on the nature of the ion. It is often supposed that this effect is due to van der Waals forces between the protein and the adsorbed molecule. Since, however, for compounds of about the same molecular weight, namely, caprylic acid, cyclohexyl

acetic acid and phenyl acetic acid, one obtains different degrees of binding with the benzene moiety less active than the paraffin moieties, this does not seem to be an adequate hypothesis.

Any organic molecule may be considered as an assembly of polar and of non-polar parts, and in aqueous solution the relation of these parts with the water will be very different. Non-polar material introduced into water produces a separation of the strongly bonded water molecules and replaces the interaction of water molecules with each other by a weaker interaction of water with the non-polar substance. Reversal of this process with recombination of the separated molecules of water results in a liberation of energy which corresponds to the energy of the surface of the non-polar material in solution. The polar parts of the molecule, in contrast, can associate with the water as well as can water with itself (Langmuir, 1925) and the adsorption of the non-polar part of the organic molecule on to or into a non-polar surface, leaving the polar parts in the aqueous phase, is therefore accompanied by a liberation of energy, since the surface of the non-polar material in contact with the water is decreased.

A semi-quantitative way of stating this is to assign to water a dipole W and to the so-called non-polar material a smaller dipole P (Gent, 1948). Before adsorption the dipole interaction energy is proportional to $2PW$ and after adsorption to $P^2 + W^2$. The change in energy is $P^2 + W^2 - 2PW$ or $(W - P)^2$ which increases as the difference between the respective dipoles increases and is always positive.

From the behaviour of the fluorescent dyes when adsorbed there is good reason to suppose that adsorption does remove the dye from the water to a second medium of lower dielectric constant, and so it seems plausible to attribute the adsorption in great part to a rejection from the water structure rather than an attraction by the protein. The concept of surface energy further accounts for the relatively low activity of a benzene moiety as compared with a paraffin, since the surface energy of benzene in water is less (34 ergs/cm.²) than for a paraffin (54 ergs/cm.²); benzene is more hydrophilic than paraffin. As would be expected, galacturonic acid, although its molecular weight is higher than most of the simple competitors included in Table 6, is ineffective in competition, as it is strongly hydrophilic and the force of rejection is weak.

In terms of the serum albumin molecule the amino-acid composition of the protein is adequate to provide the sort of surface against which these forces can act. It is visualized that the region of adsorption is one where a local excess of positive groupings is backed by a local excess of the hydro-

phobic side chains of leucine, valine and other non-polar amino-acid residues. Because of the low dielectric constant of this microphase the local charge is shielded from the general field of the protein and is not as greatly affected as it would otherwise be by variations in the net charge of the protein as a whole (Hartley, 1938). To account for the absence of corresponding sites of adsorption for positive ions, it must be supposed that the negative groups on the protein are more diffusely arranged and lack the hydrophobic backing which the positive groups have.

For each molecule the extent of adsorption will depend both on non-polar forces and attraction of negative ions. In the series of naphthylamine-sulphonic acids, for example, the addition of a sulphonic group will decrease the former and increase the latter type of interaction, the effect near the isoelectric point being to keep the total binding of these acids appreciably constant. An additional type of force may be important with bromine, iodine and the nitro group, both in inorganic ions and in organic binding. This could be a polarization in the field between the protein and the orientated dipoles of water. The low activity of the fluoride ion, as compared with the chloride, may be due to its increased hydration, keeping it in the aqueous phase by means of hydrogen bonding.

In a recent review Klotz (1949) has described his views on the special structure of the serum albumin molecule and has criticized the ideas of Davis (1943). The present work supports and extends the ideas of Davis and is in disagreement with the views of Klotz, as it underlines the importance of the surface active properties of those amino-acids residues usually regarded as inert, namely, the leucines, valine, etc. Klotz, in contrast, believes the stoichiometric relations of hydroxy amino-acids with the dicarboxylic acids to be of paramount importance. The correlations given by Klotz are nevertheless suggestive, as it is for those proteins where the hydroxyl groups are relatively less numerous than the hydrophobic backing of charged groups would be most easily achieved and the forces described in this paper most likely to be active. In view of the non-polar nature of the active surfaces on the protein and of the adsorbed molecule the idea of 'combined' water molecules being displaced on adsorption (Klotz, 1949) would seem unlikely.

The results with the dyes used in this work show that about 2-4 molecules of dye are adsorbed by each molecule of the protein at neutral pH, with the exception of eosin where more molecules combine. A decrease in the pH increases the number of sites available to negative dyes. Rosenfeld & Surgenor (1950) have found 2 molecules of ferriprotoporphyrin strongly bound to the human serum albumin molecule, while Martin (1949) has found 3 molecules

of bilirubin bound per molecule of albumin. These results contrast with the much higher values from the dialysis experiments of Klotz (1946*a*) and other workers, e.g. Teresi & Luck (1948) (10–25 molecules bound per albumin molecule). Klotz (1946*b*) has never reported these high values as a result of his spectrophotometric technique, and it may be that the dialysis technique when applied to higher dye concentration includes some type of interaction between the dye and the protein unlike that occurring at lower concentrations and measured by the present method. Certainly the low values are more in accord with the view that the effect of the positive groups is reinforced as described in this paper in a few special patches on the protein, while the higher values accord better with the view of Klotz that the amino groups need only to be freed from hydrogen bonding with other amino-acid residues in order to be active. The results of a comparison with Weber's work on covalently coupled dyes, discussed above, strongly support the idea of a special patch of positive groupings as opposed to a general activation.

Whether a similar active region is important in cases of interactions of a more specific nature requires further evidence to decide. It is likely that the forces related to surface tension described here play a part in all interactions of non-polar material in aqueous solution, and the forces of van der Waals should therefore not be used indiscriminately to account for these interactions. The analogy between the electronic state of the dye molecules at the protein surface and in organic media should be borne in mind when attempting to formulate a theory of enzyme activation.

SUMMARY

1. A method is described for studying quantitatively the adsorption of fluorescent dyes by macromolecules using measurements of the polarization and intensity of the fluorescent light. The method is applied to the adsorption of dyes by bovine serum albumin.

2. The effect of pH on the adsorption is discussed. Negatively charged dye ions are adsorbed even when the net charge on the protein is negative. A positive charge on the dye molecule inhibits combination on either side of the isoelectric point. Basic dyes are adsorbed as the free base but not as the positive ion.

3. Free carboxyl, sulphonic, hydroxyl or amino groups in the dye are not necessary for combination between dye and protein to occur. Bromine atoms increase the extent of adsorption.

4. Addition of related but non-fluorescent compounds can decrease the adsorption of a fluorescent dye by competition. Competition is also found between the dye and a variety of simple inorganic and organic ions. The activity of univalent negative inorganic ions is in the order of the Hofmeister series and that of univalent negative organic ions in the order of surface activity. Positive ions do not compete with the dyes.

5. Characteristic changes in the absorption spectra and in the fluorescence of the dyes occur on adsorption. These changes can be reproduced if the dyes are adsorbed by cetyltrimethylammonium bromide micelles or dissolved in 90 % dioxan.

6. The significance of these results is discussed and a comparison made between the adsorbed dye and the dye coupled covalently to serum albumin by the methods of Dr G. Weber. It is concluded that the results of this work can best be explained by the presence in serum albumin of a few regions of surface where a local excess of positively charged amino-acid residues is backed by residues with non-polar side chains.

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APPENDIX

Derivation of equations

Formulae (1) are derived as follows: consider a solution of dye with fluorescent intensity I_0 and polarization P_f to which protein is added. As the concentration of protein increases the fraction of dye bound to the protein increases. A small addition of protein will remove an amount of dye δx , of fluorescent intensity $I_0 \delta x$, from solution. Attached to the protein this amount of dye will have a different intensity $\lambda_x I_0 \delta x$, say, where λ_x may vary during the course of a large addition of protein. It will also have a polarization P_B larger than P_f . When a fraction x of the dye is bound the intensity of the mixture I will be

$$I = I_0 - \int_0^x I_0 dx + \int_0^x \lambda_x I_0 dx$$

$$= I_0 \left(1 - x + \int_0^x \lambda_x dx \right). \quad (i)$$

The polarization p is the average for free and bound dye weighted according to their respective intensities, i.e.

$$p = \frac{(1-x) P_f + \int_0^x P_B \lambda_x dx}{1-x + \int_0^x \lambda_x dx}. \quad (ii)$$

With P_B not dependent on x , the integral $\int_0^x \lambda_x dx$ is eliminated from (ii) using (i). Calling

$$P/P_B = \bar{p}, \quad P_f/P_B = \Pi, \quad I/I_0 = R;$$

$$\bar{p} = \frac{(1-x) \Pi + R + x - 1}{R}, \quad (iii)$$

$$\text{or} \quad (1 - \Pi) x = \bar{p} R - R + 1 - \Pi. \quad (1b)$$

Equations (1) and (1a) are derived from this by putting $P_f = \Pi = 0$ and $R = 1$ progressively.

Formula (2) is based on a simple proportionality argument. Formula (4) is derived as follows: Suppose the fluorescence is excited by light plane polarized at an angle θ to the axis of observation and has a polarization p . The observed intensity I' will be the sum of three components, namely,

$$I' = I_1 \sin^2 \theta + I_2 \cos^2 \theta + I_3, \quad (iv)$$

where I_1 and I_2 are the intensities of the components of fluorescence parallel and perpendicular to the exciting vector and so $p = \frac{I_1 - I_2}{I_1 + I_2}$ by definition, or $I_2 = \frac{(1-p)}{(1+p)} I_1$. Unpolarized excitation is equivalent to averaging for all angles θ or since $\overline{\sin^2 \theta} = \overline{\cos^2 \theta} = \frac{1}{2}$;

$$I' = \frac{1}{2} I_1 + \frac{1}{2} I_2$$

$$= I_1 \left(\frac{1}{2} + \frac{3}{2} \frac{1-p}{1+p} \right)$$

$$= I_1 \left(\frac{2-p}{1+p} \right). \quad (v)$$

For excitation perpendicular to the direction of observation on the other hand $\theta = 90^\circ$, and

$$I = I_1 + I_2$$

$$= I_1 \left(1 + \frac{1-p}{1+p} \right)$$

$$= I_1 \left(\frac{2}{1+p} \right). \quad (vi)$$

Combining (v) and (vi)

$$I = \frac{I'}{1 - \frac{1}{2} p}. \quad (4)$$