Spectrophotometric Studies of the Binding of Trypan Blue to Bovine Serum Albumin*

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ABSTRACT: An analysis is presented showing that qualitative spectral changes incident to binding can be interpreted in terms of sets of binding sites. A set is defined as a number of occupied sites which are individually indistinguishable spectroscopically, and the minimum number of sets is equal to the number of qualitative spectral changes. Occupied sites will belong to the same set if they have identical spectral properties, identical association constants, or if cooperative binding occurs between them. Interactions may alter sites so that a set may cease to exist as additional sites are filled and a new set is formed. The spectra of solutions of the azo dye trypan blue and bovine serum albumin have been measured at pH 7.4 and 3.3 over a wide range of dye to protein ratios. At pH 7.4 the data indicate that there are two sets and a total of three binding sites. The model most consistent with the results is one in which the stronger set comprises two sites, and the filling of the third site involves interactions that result in the disappearance of the stronger set with the formation of the weaker set, which then consists of all three sites. At pH 3.3 and high dye to protein ratios an insoluble complex is formed having approximately the composition PD_{31}. At relatively lower dye to protein ratios soluble complexes appear, and the soluble complex in equilibrium with the insoluble one is PD_9.

There are three sets of sites in the soluble complexes, and the two strongest sets together consist of at least five sites.

A preliminary study of the effect of BSA[1] on the spectrum of trypan blue revealed unusual properties of the system, and it was decided to investigate the interactions in detail. Of special interest are the facts that more than one spectroscopically distinguishable complex is formed and that the binding is relatively specific in the sense that there is a very small number of binding sites. This latter characteristic would make the BSA–trypan blue system potentially useful for investigating the nature of the binding sites, although that is not the subject of this communication. In what follows the meaning of spectral changes consequent to binding are examined and the concepts arrived at are applied to the BSA–trypan blue reactions.

Materials and Methods

Crystallized BSA (lot no. A69805) was obtained from Armour Pharmaceutical Co. BSA solutions were passed through a bacterial filter, which substantially reduced the turbidity in the visible spectrum. Concentrations were calculated from the measured optical density at 278 mμ using the values ϵ 0.66 (mg/ml)$^{-1}$ cm^{-1} and 69,000 for the molecular weight.

Trypan blue (Colour Index Direct Blue 14, 3,3′-[(3,3′-dimethyl-4,4′-biphenylene)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid]) was an Allied Chemical Corp. product. It was purified by precipitation from hot aqueous solution by addition of four volumes of ethanol (Hartwell and Fieser, 1936). After three precipitations a red impurity was no longer detectable chromatographically. A portion of the purified product was dried to constant weight at 105° and dissolved in water. On the assumption that the product was 100% pure the molar absorbancy was found to be 6.40×10^4 M^{-1} cm^{-1} at λ 600 mμ; in 0.1 ionic strength Tris-NaCl buffer ϵ_{585} 5.81×10^4 M^{-1} cm^{-1}.

There was a slow deterioration of trypan blue solutions, as evidenced by the appearance of a red impurity. The process was considerably faster in acetate buffer at low pH than in neutral solutions. A convenient purification procedure was devised for the preparation of fresh stock solutions which took advantage of the strong adsorption of trypan blue to cellulose from dilute salt solutions and the lack of adsorption from distilled water (Kelly, 1958). To a column packed with surgical cotton, previously washed with 0.1 M NaCl, was added about 0.8 dead column volume of 0.1% trypan blue in 0.1 M NaCl. After allowing about 3 hr for adsorption of the dye, the column was washed with 0.1 M NaCl, which eluted a red impurity, and then with about three column volumes of distilled water, which removed a small part of the trypan blue. After standing with water overnight to allow time for desorption, elution with distilled water was continued, and the more concentrated fraction, having $A_{600} \sim 20$–30, was saved for use in the experiments. No red dye was

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[1] Abbreviation used: BSA, bovine serum albumin.

detected in these solutions after 3 weeks at room temperature.

Tris was a primary standard grade. All other chemicals were of reagent grade. All solutions were prepared in distilled and deionized water.

Spectra were taken with a Cary Model 14 double-beam spectrophotometer. Temperature was not closely controlled but was in the range 22–25°.

Binding at pH 7.4. A 2-ml aliquot of trypan blue solution of maximum absorbance near 1.5 in 0.1 ionic strength Tris-HCl buffer was placed in the sample cuvet equipped with a magnetic stirring bar. A portion of the identical solution was placed in the reference cuvet. After balancing the spectrophotometer to compensate for mismatching, aliquots of a BSA solution were added to the sample solution with stirring, by means of a syringe microburet. The BSA solution contained the same concentration of buffer and trypan blue as the solution to which it was being added, so that the total dye concentration was unchanged throughout the titration. After each addition of BSA the difference spectrum was recorded. In this way a series of difference spectra were obtained covering a wide range of dye to protein ratios. The protein concentration was varied from 2.4×10^{-6} to 1.6×10^{-3} M.

Binding at pH 3.3. Solutions all containing the same concentration of dye (1.30×10^{-5} M) but varying concentrations of BSA were prepared separately. The BSA was added to the dye solution with stirring. The protein concentration was varied from 2.5×10^{-7} to 2.6×10^{-5} M. The solutions were permitted to stand for 2 hr and were then centrifuged and the presence or absence of precipitate noted. Spectra were run on all supernatants. The acetate buffer was 0.09 M in NaCl and 0.01 M in sodium acetate.

Spectrum of Free Dye. At both pH 7.4 and 3.3, 0.1 ionic strength, the spectrum of trypan blue was measured at concentrations up to 3×10^{-5} M. There was a very small effect of concentration on the shape of the spectrum, especially below 5×10^{-6} M. However, the error introduced by ignoring this effect was found to be insignificant, and no correction for it has been made.

Equilibrium Dialysis. The very strong binding of trypan blue to dialysis tubing precluded the performance of equilibrium dialysis measurements with this dye.

Results and Discussion

If a dye is bound to a protein its spectrum will, in general, change (Klotz, 1953). Keeping the dye concentration constant and increasing the protein concentration in small steps, one would expect to see a gradual transition from a spectrum characteristic of free dye to one characteristic of completely bound dye. If only two spectroscopically distinguishable species were involved the change occurring at any wavelength would be in one direction only and would be quantitatively characteristic of the titration of a single function. If the curves happened to cross there would be one or more isosbestic points. More rigorously, the ratio of the absorbance changes at any two wavelengths would be the same for all solutions. This is equivalent to saying that the difference spectra, using, for instance, free dye as the reference solution, would all have the same shape.

Consider the reaction $P + D = PD_2 + \ldots + PD_n$. Assume that the absorbance of P is zero in the spectral region of interest. Let D_0 and D be the total and free concentrations of dye, P_0 the total concentration of BSA, α_i the fraction of the ith site of the BSA which is occupied by dye, ϵ_0 the molar absorbancy of free dye, ϵ_i the molar absorbancy of the dye on the ith site, and A and A_0 the absorbance of the test and reference solutions. Then, since $\alpha_i P_0$ is the concentration of the dye on the ith site, $A = D\epsilon_0 + \Sigma\alpha_i P_0 \epsilon_i$. The summation is from 1 through n. For difference spectra in which D_0 is the same for both reference and sample and protein is absent from the reference it can be shown that

$$\Delta A = P_0 \Sigma \alpha_i \Delta\epsilon_i \tag{1}$$

where $\Delta\epsilon_i = \epsilon_i - \epsilon_0$. For measurements at two wavelengths λ and λ'

$$\Delta A/\Delta A' = \Sigma\alpha_i \Delta\epsilon_i / \Sigma\alpha_i \Delta\epsilon_i' \tag{2}$$

As pointed out above, $\Delta A/\Delta A'$ may be used to define the shape of difference spectra. For a series of solutions with constant D_0 and varying P_0, the shapes of the spectra at the different values of P_0 will be identical if and only if for any two wavelengths $\Delta A/\Delta A'$ is the same for all spectra. The α_i are functions of P_0. $\Delta A/\Delta A'$ will be independent of P_0 for the following three models.

(a) All the α_i are equal. Then eq 2 becomes $\Delta A/\Delta A' = \Sigma\Delta\epsilon_i/\Sigma\Delta\epsilon_i'$. This will be true if the sites all have the same intrinsic binding constant and if any interactions between sites affect all sites equally (Tanford, 1961), and also for the limiting case where all $\alpha_i = 1$, *i.e.*, where protein is completely saturated with dye. (b) There is cooperative binding, such that all n sites on any given protein molecule are occupied, or none are. Then $A = D_0\epsilon_0 + \gamma P_0 \Sigma\epsilon_i$, where γ = fraction of the protein molecules occupied. For this case also $\Delta A/\Delta A' = \Sigma\Delta\epsilon_i/\Sigma\Delta\epsilon_i'$. (c) All of the occupied sites have identical spectra, *i.e.*, $\Sigma\epsilon_i = n\epsilon$. It can then be shown that $\Delta A/\Delta A' = \Delta\epsilon/\Delta\epsilon'$.

Since the expressions for $\Delta A/\Delta A'$ are independently derived, the noteworthy conclusion follows that if $\Delta A/\Delta A'$ does change with change in P_0 (the shapes of the difference spectra change), then none of the three models can be true of the system. Conversely, if the shape of the curves does not depend on P_0, then each of the models (or more elaborate combinations of them) is a possibility, and a choice would require auxiliary information.

For a particular protein–small molecule system there may be identical and nonidentical binding

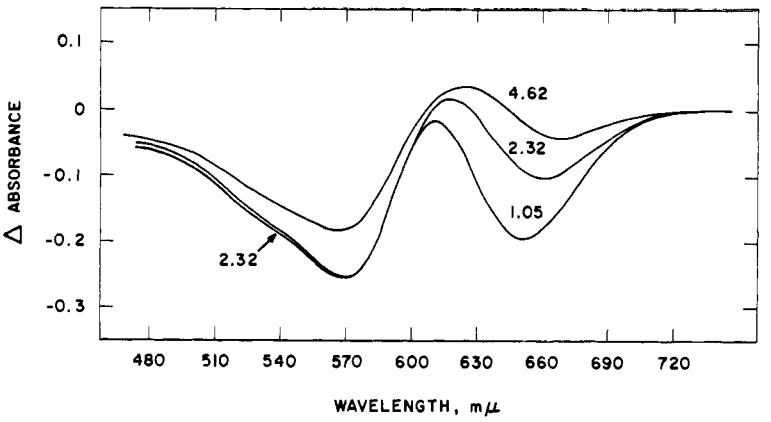

FIGURE 1: Difference spectra at pH 7.4 for the trypan blue to BSA ratios indicated. Trypan blue concentration, 2.56×10^{-5} M.

sites. Defining a set as a number of occupied sites which are spectroscopically indistinguishable, a count of the number of qualitative spectral changes provides a count of the minimum number of sets of sites which are distinct in both their spectral properties and their binding constants. It is recognized, however, that differentiation of sites which are close to each other in either or both of these properties will be limited by the precision of the measurements and the rigor of the analysis. Furthermore, interactions may induce changes in occupied sites as additional sites are filled. Therefore, although a spectral change implies the appearance of a new set, part of the change may be due to alterations in existing sets with loss of their original identity. Changes in the properties of individual occupied sites or sets will always be a possible concomitant of a spectral change and will not be directly detectable, because the measured difference spectrum is the concentration-weighted sum of the difference spectra of all occupied sites (eq 1). In favorable cases, however, the occurrence or nonoccurrence of interactions may be inferred from an analysis of the concentration dependence of absorbance changes, as will be illustrated.

In terms of sets the equation for the absorbance of a solution of dye and protein may be rewritten as $A = D\epsilon_0 + P_0\Sigma n_j\bar{\alpha}_j\bar{\epsilon}_j$ and the absorbance difference as

$$\Delta A = P_0\Sigma n_j\bar{\alpha}_j\Delta\bar{\epsilon}_j \qquad (3)$$

for m sets consisting of $n_1, n_2, \ldots, n_m$ sites. $\bar{\alpha}_j$ is the bound fraction of the set, $\bar{\epsilon}_j$ is the average absorbancy of the dye on the sites within the set, and $\Delta\bar{\epsilon}_j = \bar{\epsilon}_j - \epsilon_0$.

The binding of trypan blue to BSA is a case where more than one spectral change occurs. Figure 1 illustrates some difference spectra at pH 7.4, 0.1 ionic strength Tris-HCl buffer. It can readily be seen that there are qualitative as well as quantitative changes which depend on the relative amounts of dye and BSA. The maximum near 620 mμ became positive with the first increments of BSA and then became negative, with a wavelength shift. Similarly, there was a substantial shift in the longer wavelength minimum. No significant qualitative change occurred, however, in the vicinity of the 570-mμ minimum, implying that in this region the spectra of all bound species were identical or very similar.

A convenient way to summarize the data from the difference spectra is to read off the absorbance differences, ΔA, at selected wavelengths and plot against the dye to protein ratio, D_0/P_0. Such plots are shown in Figure 2. Correction has been made for the absorbance due to the BSA solution. It is evident that in general $\Delta A/\Delta A'$ varies with D_0/P_0. Considering first the $D_0/P_0 > 0.3$ portion of the curves, there is one readily apparent change in both the ΔA_{620} and ΔA_{650} curves: as P_0 increased ΔA_{620} at first became positive and then negative, and ΔA_{650} at first remained near zero and then became rapidly negative. Also ΔA_{620} and ΔA_{650} were still changing after ΔA_{570} had reached a constant value. In the high range of D_0/P_0 there are then two sets of sites distinct in both their spectral characteristics and their binding constants.

At higher concentrations of BSA ($D_0/P_0 < 0.3$) another change in the difference spectra ensued, and at the highest concentration employed, 1.6×10^{-3} M, there was no sign of leveling off. An experiment was performed in which the same high range of protein concentrations was covered but at about one-third the dye concentration, and when the data of both experiments were plotted as $\Delta A/D_0$ *vs.* P_0 it was found that all the points fell on the same curve. The change was due, therefore, to the BSA concentration *per se* and was independent of the dye to BSA ratio. The reason for this is not clear, but it may be due to the fact that the BSA has become an appreciable portion of the solvent in this range, or it may imply some aggregation of the dye–BSA complex. Aggregation has been suggested as a possible explanation of the

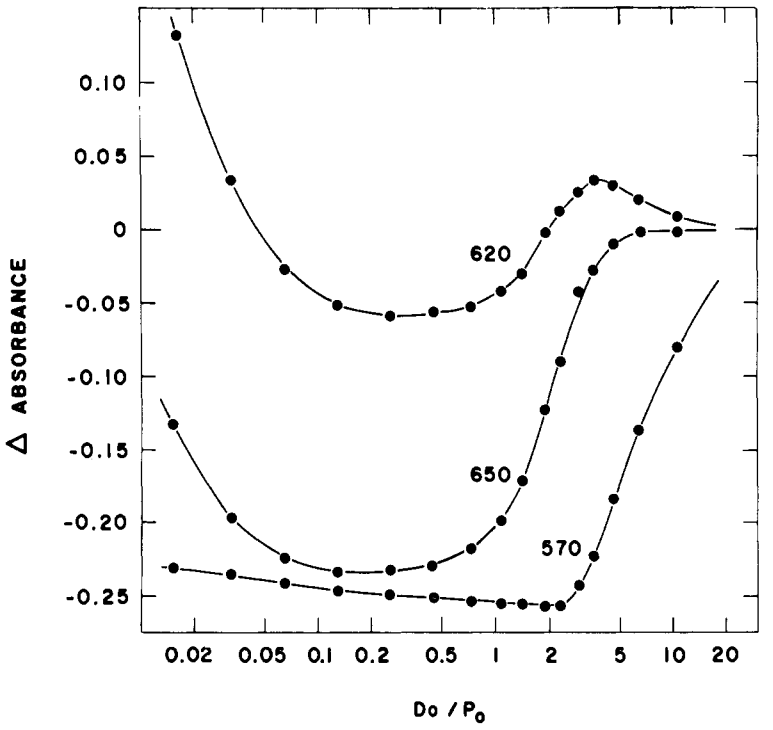

FIGURE 2: Absorbance difference *vs.* trypan blue to BSA ratio at 570, 620, and 650 mμ. Trypan blue concentration, 2.56×10^{-5} M, pH 7.4.

TABLE I: Expressions for the Fractions of the Protein Present as PD, PD_2, and PD_3 for Different Models.

Case	PD/P_0	PD_2/P_0	PD_3/P_0
1a and 3a	$\beta_1\frac{D_0}{P_0}$	$3\left(1-\beta_1\frac{D_0}{P_0}-\frac{1}{3}\beta_2\frac{D_0}{P_0}\right)$	$\beta_2\frac{D_0}{P_0}+2\beta_1\frac{D_0}{P_0}-2$
2a and 4a	$2-\beta_1\frac{D_0}{P_0}-\frac{2}{3}\beta_2\frac{D_0}{P_0}$	$\beta_1\frac{D_0}{P_0}+\frac{1}{3}\beta_2\frac{D_0}{P_0}-1$	$\frac{1}{3}\beta_2\frac{D_0}{P_0}$
1b and 3b	$\beta_1\frac{D_0}{P_0}$		$\frac{1}{3}\beta_2\frac{D_0}{P_0}$
2b and 4b		$\frac{1}{2}\beta_1\frac{D_0}{P_0}$	$\frac{1}{3}\beta_2\frac{D_0}{P_0}$

effect of protein concentration on the binding of dodecyl sulfate (Ray *et al.*, 1966).

If it is assumed, as seems reasonable from the spectra, that at 570 mμ the absorbancy of the dye is substantially the same in all its complexes, then $\bar{\nu}$, the average number of molecules of trypan blue bound per molecule of BSA, can be calculated with the equation $\bar{\nu} = (\Delta A/\Delta A_{max})_{570} \times (D_0/P_0)$, and the number of sites, n, can then be estimated since $\bar{\nu} \rightarrow n$ as $P_0 \rightarrow 0$. The values of $\bar{\nu}$ so calculated converged rapidly to 3.3. Since the nominal concentrations of dye and BSA are most likely to err on the high side and the low side, respectively, the value of 3 for the number of sites is favored. Allen and Arahovats (1950) found $n = 2$ by a method involving competition between BSA and cellulose for trypan blue.

Additional information of interest can be derived from the ΔA_{650} *vs.* D_0/P_0 curve. At higher D_0/P_0 values, where the calculation of $\bar{\nu}$ from the ΔA_{570} curve indicates that all sites are essentially occupied, $\Delta A_{650} \approx 0$. Writing eq 3 for two sets of sites, $\Delta A = P_0(n_1\bar{\alpha}_1\Delta\bar{\epsilon}_1 + n_2\bar{\alpha}_2\Delta\bar{\epsilon}_2)$, it is evident that for ΔA to equal zero when the protein is saturated, either the two terms must cancel or they must both become zero. That is, either $n_1\Delta\bar{\epsilon}_1 = -n_2\Delta\bar{\epsilon}_2$ or $\bar{\alpha}_1 = 0$ and $\Delta\bar{\epsilon}_2 = 0$. The latter case means that there are interactions and/or configura-

tional changes such that the stronger set disappears as additional sites are filled and the weaker set consists of the total of all sites. Given the constraint that ΔA_{650} goes to zero as the sites are filled, the values that $\Delta\bar{\epsilon}_2/\Delta\bar{\epsilon}_1$ can have are $-1/2$ (for $n_1 = 1$, $n_2 = 2$), -2 (for $n_1 = 2$, $n_2 = 1$), and 0 (for $n_2 = 3$).

At $\lambda = 570$, $\Delta\bar{\epsilon}_1 = \Delta\bar{\epsilon}_2 \equiv \Delta\epsilon$ and $\Delta A = P_0\Delta\bar{\epsilon}(n_1\bar{\alpha}_1 + n_2\bar{\alpha}_2)$. When all the dye is bound the absorbance difference will be maximal: $A_m = D_0\Delta\bar{\epsilon}$. Likewise, at wavelengths for which $\Delta\bar{\epsilon}_1 \neq \Delta\bar{\epsilon}_2$, when all the dye is bound to the *stronger* set ($\bar{\alpha}_2 = 0$) the absorbance difference will be maximal: $A_m = D_0\Delta\bar{\epsilon}_1$. Then

$$\left(\frac{\Delta A}{\Delta A_m}\right)_{570} = \frac{P_0}{D_0}(n_1\bar{\alpha}_1 + n_2\bar{\alpha}_2) = \beta_1 + \beta_2 \qquad (4)$$

and

$$\left(\frac{\Delta A}{\Delta A_m}\right)_{650} = \frac{P_0}{D_0}\left(n_1\bar{\alpha}_1 + n_2\bar{\alpha}_2\frac{\Delta\bar{\epsilon}_2}{\Delta\bar{\epsilon}_1}\right) = \beta_1 + \beta_2\frac{\Delta\bar{\epsilon}_2}{\Delta\bar{\epsilon}_1} \qquad (5)$$

where β_1 and β_2 are the fractions of dye bound to the stronger and weaker sets. From the data and these equations sets of values of β_1 and β_2 can be calculated for each of the three values of $\Delta\bar{\epsilon}_2/\Delta\bar{\epsilon}_1$. Finally, equations for the fraction of protein in each of the complexes PD, PD_2, and PD_3 can be derived in terms of the β's. Several cases may be distinguished: (1a) $n_1 = 1$, $n_2 = 2$ equivalent sites; (1b) $n_1 = 1$, $n_2 = 2$ cooperative sites; (2a) $n_1 = 2$ equivalent sites, $n_2 = 1$; (2b) $n_1 = 2$ cooperative sites, $n_2 = 1$; (3a) $n_1 = 1$, $n_2 = 3$ equivalent sites; (3b) $n_1 = 1$, $n_2 = 3$ cooperative sites; (4a) $n_1 = 2$ equivalent sites, $n_2 = 3$; (4b) $n_1 = 2$ cooperative sites, $n_2 = 3$. $\Delta\bar{\epsilon}_2/\Delta\bar{\epsilon}_1 = -1/2$ for 1a and b, -2 for 2a and b, and 0 for the others. In this context equivalent is used to mean indistinguishable spectroscopically because of equal association constants or equal spectra of the occupied sites, as discussed above.

The equations for the fraction of each complex are shown in Table I and the calculated values are given in Table II. The equations were derived with the assumption that the fraction of free protein is negligibly small. The data used in the calculations were those at $D_0/P_0 = 3.0$ and higher, for which the assumption seems reasonable. It should be noted that for those pairs of cases for which the equations are identical the β's are different. For cases 1a, 2a, 3a, and 4a the sums of the expressions themselves are equal to 1; therefore, no matter what the values of β_1 and β_2, the sum of the values of the fractions PD_i/P_0 will necessarily be equal to 1 and the sum will not serve to test the validity of the model. A model might be eliminated, however, on the basis of untenable individual values, since physically $0 \leq PD_i/P_0 \leq 1$. For the remaining cases the expressions do not sum 1; therefore, the sums of the values of the fractions can serve as a test of validity, along with the criterion that $0 \leq PD_i/P_0 \leq 1$. In Table II it can be seen that case 4b is the most compatible with the data, since there are no negative fractions and the sum is close to 1. If a positive 10% error in D_0/P_0 values is assumed, the fit for case 4b is improved, and case 4a would then also be tenable. The data, therefore, support the assignment of two sites to the stronger set and interactions incident to the filling of the third site such that the stronger set disappears and the weaker set consists of all three sites. A choice between equivalent or all-or-none cooperative sites for the stronger set cannot be made within a reasonable expectation of

TABLE II: Test of the Three-Site Two-Set Models.

D_0/P_0	PD/P_0	PD_2/P_0	PD_3/P_0	$\Sigma PD_i/P_0$
		Case 1a		
4.6	1.238	−2.799	2.562	
4.0	1.296	−2.796	2.500	
3.5	1.327	−2.730	2.404	
3.0	1.341	−2.517	2.176	
		Case 1b		
4.6	1.238		0.695	1.933
4.0	1.296		0.636	1.932
3.5	1.327		0.583	1.910
3.0	1.341		0.498	1.839
		Case 2a		
4.6	−0.976	1.629	0.347	
4.0	−0.886	1.569	0.317	
3.5	−0.786	1.494	0.292	
3.0	−0.586	1.337	0.249	
		Case 2b		
4.6		1.141	0.347	1.488
4.0		1.126	0.317	1.443
3.5		1.101	0.292	1.393
3.0		1.044	0.249	1.293
		Case 3a		
4.6	0.199	−0.720	1.521	
4.0	0.344	−0.891	1.548	
3.5	0.452	−0.981	1.529	
3.0	0.594	−1.023	1.429	
		Case 3b		
4.6	0.199		1.041	1.240
4.0	0.344		0.953	1.297
3.5	0.452		0.875	1.327
3.0	0.594		0.747	1.341
		Case 4a		
4.6	−0.281	0.240	1.041	
4.0	−0.250	0.297	0.953	
3.5	−0.202	0.327	0.875	
3.0	−0.088	0.341	0.747	
		Case 4b		
4.6		0.099	1.041	1.140
4.0		0.172	0.953	1.125
3.5		0.226	0.875	1.101
3.0		0.297	0.747	1.044

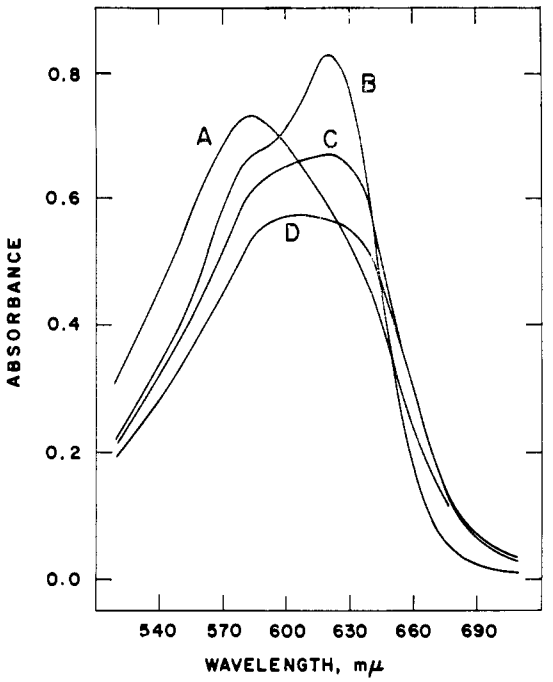

FIGURE 3: Spectra of trypan blue at pH 3.3. Curve A, no BSA; curve B, $D_0/P_0 = 0.5$; curve C, $D_0/P_0 = 5$; curve D, $D_0/P_0 = 8$. Trypan blue concentration, 1.30×10^{-5} M.

error. Some aspects of the binding of dodecyl sulfate to BSA were similarly interpreted as due to a loss of strong sites as weaker ones were filled (Pallansch and Briggs, 1954; Foster, 1960).

Binding at pH 3.3. At pH 3.3 and high dye to protein ratios, D_0/P_0, the interaction was characterized by the appearance of an insoluble product. Progressively greater amounts of dye were removed from the solutions as the amount of BSA was increased, until the supernatant was colorless. The spectra of the supernatants up to this point were identical with that of the dye in the absence of BSA. As D_0/P_0 was further decreased, part of the dye remained in solution and the amount of insoluble product progressively diminished. The spectra of the supernatants of these solutions were qualitatively indistinguishable from each other throughout the range in which precipitate was found, but they were distinct from the spectrum of free dye. At the point of incipient formation of the precipitate D_0/P_0 was about 8. Further lowering of D_0/P_0 resulted in continuous changes in the spectra down to $D_0/P_0 = 0.5$, below which there was no more change. The spectra of some of the solutions are shown in Figure 3. Curve A is the spectrum of free dye and the curves labeled B, C, and D are spectra of the $D_0/P_0 = 0.5$, 5, and 8 solutions, respectively. There was an isosbestic point for the spectra of all the solutions between $D_0/P_0 = 0.5$ and 5 at about 642 mμ, but the spectra of the $D_0/P_0 = 5$–8 solutions did not cross at this point. From this it appears that the spectrum C did not result simply from a suitable mixture of dye of spectra B and D, but rather that there are three qualitatively distinguishable spectra and therefore there are three distinct sets of sites, differing in both their association constants and spectra.

In the range of precipitation where the supernatants contained only free dye, the composition of the insoluble complex, PD_t, calculated with the equation $t = (D_0 - D)/P_0$, was found to be constant over the entire range, at about PD_{31}. The precipitate dissolved slowly in buffer at pH 7.4, or at pH 3.3 in the presence of excess BSA, demonstrating that its formation was reversible.

For the range of precipitation where the supernatants contained bound dye, assuming that a single soluble complex, PD_s, was in equilibrium with a single insoluble complex, PD_t, and that the concentrations of free dye and BSA were negligible, eq 6 was derived.

$$\frac{sPD_s}{D_0} = \frac{st}{t - s}\frac{P_0}{D_0} - \frac{s}{t - s} \qquad (6)$$

sPD_s/D_0 was set equal to A/A_0, where A_0 was the absorbance of the $D_0/P_0 = 8$ solution. A plot of A/A_0 *vs.* P_0/D_0 was a satisfactory straight line, and from its slope and intercept the values $s = 9.2$ and $t = 27$ were calculated. When the value $t = 31$ found from the dye excess region was introduced as an additional point, s was nearly unchanged at 9.1. The soluble complex in equilibrium with the insoluble complex is taken to be, therefore, PD_9. Apparently the formation of the insoluble compound PD_{31} from PD_9 is a strongly cooperative phenomenon, probably involving a considerable opening out of the protein molecule.

For the soluble complexes of trypan blue and BSA at pH 3.3 there are nine sites distributed among three sets. That the dye is very tightly bound to the protein is shown by the fact that near the equivalence point of the insoluble complex the dye is not detectable in the supernatant solution. Therefore, the average number of dye molecules bound per molecule of protein is essentially equal to D_0/P_0. The limiting spectrum at low D_0/P_0 is that of the first, or strongest, set. The change in the spectrum at $D_0/P_0 > 0.5$ indicates that the weaker sets are being occupied. However, it is evident from the existence of the isosbestic point up to $D_0/P_0 = 5$ that the third set is not appreciably occupied in this range. It can be inferred, therefore, that the sum of the sites in the first two sets must be at least 5. Since it is unlikely that the association constants for the third set are so much smaller than those of the others that the latter will be completely filled before the former are detectable, it is reasonable to assume that the number of sites in the first two sets is actually greater than 5.

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The Phosphoprotein of the Dentin Matrix*

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ABSTRACT: Purified, decalcified bovine dentin matrix contains a small amount of bound phosphorus but otherwise appears to have a composition typical of mammalian collagens. The bound phosphorus cannot be removed by nonhydrolytic or other nondegradative extractions. Prolonged oxidative degradation of the matrix with sodium metaperiodate resulted in the solubilization of about 30% of the matrix and this portion contained more than 75% of the phosphorus. Fractionation of the solubilized dentin led to the isolation of a rapidly moving, electrophoretically homogeneous anionic component (designated F component) at pH 5.3. Equilibrium ultracentrifuge studies showed the F component to be homogeneous with respect to molecular weight and to have a molecular weight of 38,000 ± 3000. Analyses showed F to be a protein, containing unusually large amounts of serine and aspartic acid plus smaller amounts of proline, hydroxyproline, and one residue of hydroxylysine per molecule. F contained 5.9% P by weight of 34 phosphate groups/molecule. Since hydroxylysine is destroyed under the conditions of periodate degradation unless either the hydroxyl or ε-amino group is blocked, and since the F component contained almost all of the hydroxylysine of the dentin which survived the periodate degradation it was concluded that the F component phosphoprotein is attached to the collagen matrix *via* the hydroxylysine side chain. The phosphoprotein comprises less than 2% of the total matrix on a weight basis or less than one molecule per four molecules of collagen. This highly anionic phosphoprotein, bound to the collagen matrix, may provide the sites for the epitactic nucleation of mineralization of the matrix.

The collagen of the dentin matrix has several characteristics which distinguish it from the more generally studied soft tissue collagens. The most distinctive properties of dentin matrix collagen are those of extreme resistance to dimensional change and an equally marked resistance to solubilization by all except degradative treatments. There is also, in contrast to bone matrix, a much lower rate of turnover. Glimcher and Katz (1965) have attributed these properties to an exceptionally strong interaction between neighboring molecules, forced upon the system when mineralization depletes the water content of the tissue. The peculiar matrix organization and fibrillar weave, clearly seen at the electron microscopic level (A. Veis and O. O. Mussell, unpublished results), may contribute to the structural stability. Veis and Schlueter (1964), however, proposed that the limited solubility might be the result of an especially high degree of intermolecular covalent cross-linking, in which an extra set of polymerizing bonds were superimposed on the structure in addition to the normal soft-tissue polymerization system (Schlueter and Veis, 1964). Although analytical data on the composition showed dentin collagen to be very similar to purified soft tissue collagen in terms of carbohydrate and amino acid components (Eastoe, 1963; Piez, 1961; Veis and Schlueter, 1964) one distinct difference was the presence of covalently bound phosphate in the completely demineralized dentin (Veis and Schlueter, 1964; McKernan and Dailly, 1966). While the presence of the phosphate might be an unimportant artifact, as suggested by Glimcher and Krane (1964), compact bone matrix collagen, also a highly mineralized tissue in which the matrix collagen is exposed to relatively high phosphate concentrations, contains much less covalently bound phosphate (McKernan and Dailly, 1966), and phosphate appears in only trace amounts in soft-tissue collagens (Glimcher *et al.*, 1964). Thus, it appeared to us reasonable to suppose that the bound phosphate played some definite role in the dentin matrix, in par-

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