[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Binding of Organic Ions by Proteins. Charge and pH Effects

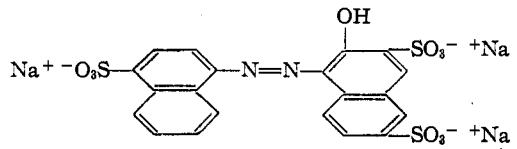
BY IRVING M. KLOTZ AND F. MARIAN WALKER

It has been shown recently[1] that quantitative data on the binding of monovalent and divalent organic anions by proteins can be correlated very well by equations derived from the law of mass action, if deviations from statistical behavior are attributed to electrostatic interactions. As a further application of this theoretical approach it has seemed desirable, therefore, to extend these equations to a trivalent anion. The results obtained are described in this paper.

The binding of organic anions by proteins may be studied also by investigating changes in absorption spectra.[2] Though this method is limited in precision at high dye concentrations, it has the advantage that data may be obtained very rapidly. Consequently it is particularly suitable for investigations of binding in alkaline solution, for at high *p*H's the dialysis–equilibrium method,[1] extending as it does over a number of days, introduces the uncertainty of possible denaturation of the protein. The spectral method has been used, therefore, to investigate the effect of *p*H on the binding of some organic ions by bovine serum albumin.

Experimental

Reagents.—The crystalline bovine serum albumin was the same as that used previously.[1,2] All of the dyes, except amaranth, have also been described in previous work. Amaranth (FD and C Red No. 2) a tri-sulfonated dye with the structural formula



was obtained through the courtesy of the National Aniline Division of the Allied Chemical and Dye Corporation and had a purity of 90.0%.

Absorption Spectra.—The absorption of light by the dye solutions was determined with the Beckman spectrophotometer in a room at approximately 25°. One-centimeter cells were used.

Dialysis Experiments.—The procedure was the same as that described previously.[1]

Ultrafiltration Experiments.—Ten cc. of the albumin-dye solution was placed in a cellophane bag, attached to a tube whose other end was open to the atmosphere, and ultrafiltered under a pressure difference of 15 cm. of mercury. Approximately 2 cc. of filtrate was collected in three hours and was then analyzed colorimetrically.

Results and Discussion

Effect of Charge on Dye.—The data on the binding of amaranth by bovine serum albumin at *p*H 5.7 are summarized in Fig. 1. For purposes of comparison, the corresponding curves for methyl orange and for azosulfathiazole, monosulfonated and disulfonated dyes, respectively, are also illustrated. It is immediately obvious that there is a progressive decrease in curvature as one proceeds from the monovalent to the trivalent anion. The degree of curvature is an expression of the electrostatic repulsion between successively bound ions. The greater the repulsion the fewer is the number of bound ions and hence the smaller is the curvature.

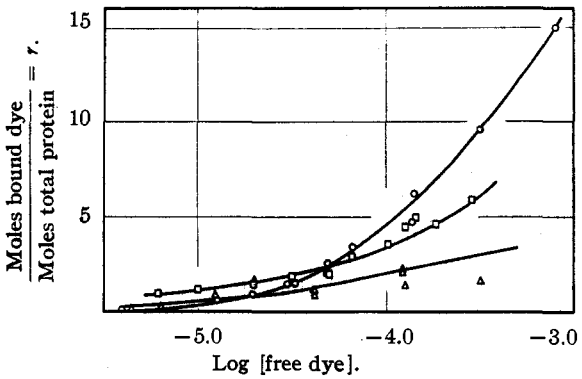


Fig. 1.—Binding of sulfonate compounds by bovine serum albumin at *p*H 5.7: ○, methyl orange (monovalent); □, azosulfathiazole (divalent); △, amaranth (trivalent).

It has been shown previously[1] that these qualitative considerations may be expressed in quantitative form by the following procedure. Recognizing that a single protein molecule may bind many ions, we express the equilibria involved in terms of equations of the general form

$$PA_{i-1} + A = PA_i \qquad (1)$$

P represents the protein, A, the anion and i may be any integer from 1 to n, the maximum number of bound anions. The moles of bound anion per mole of total protein, represented by r, is obtained from the expression

$$r = \frac{\sum_{i=1}^{n} i\left(\prod_{j=1}^{i} k_j\right)(A)^i}{1 + \sum_{i=1}^{n}\left(\prod_{j=1}^{i} k_j\right)(A)^i} \qquad (2)$$

where the k's represent the equilibrium constants corresponding to each of the reactions of (1). Thus the calculation of the moles of bound anion per mole of total protein depends on a knowledge of these n equilibrium constants.

For the ideal situation of no electrostatic interaction, the equilibrium constants bear a simple relation to each other, given by the equation

$$k_i = \frac{n - (i-1)}{i} k \qquad (3)$$

k is an intrinsic constant which depends on the

(1) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(2) I. M. Klotz, *ibid.*, **68**, 2299 (1946).

nature of the protein and of the anion and is determined empirically. Where electrostatic interactions cannot be neglected, successive equilibrium constants may be related to each other by the expression

$$RT \ln (k_{i-1}/k_i) = RT \ln \left[\frac{n - (i-2)}{n - (i-1)} \frac{i}{i-1}\right] + \frac{Nz^2e^2}{D}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \quad (4)$$

where N is Avogadro's number, z, the number of charges on A, e, the electronic charge, D, the dielectric constant of the medium, b, the radius of the protein molecule, a, the "distance of closest approach" of a charged ion to the protein and κ is given by

$$\kappa = \left(\frac{4\pi Ne^2}{1000DKT}\right)^{1/2} \Gamma^{1/2} \quad (5)$$

where K is the Boltzmann constant, T, the absolute temperature and Γ twice the ionic strength of the medium. In using equation (4), one is assuming implicitly that the protein molecule may be approximated by a spherical model and that the electrostatic interactions are expressed sufficiently well by the Debye–Hückel theory. It must be recognized, of course, that the actual situation in the solution is much more complex and that further experiments may require a much more refined theoretical framework.

To calculate the electrostatic-interaction term for amaranth, it was assumed as in previous work[1] that the radius, b, of the serum albumin molecule in solution is 30 Å. From a model of the amaranth molecule, an estimate of 8.5 Å. was made for the average radius to the sulfonate groups. Knowing a and b and the ionic strength (0.120), one may calculate the electrostatic term in (4) and hence also the ratio of successive equilibrium constants. The intrinsic constant, k, of amaranth was evaluated empirically by the procedure previously described.[1] Values of k_i were then determined and the calculated curve for r was obtained. The result is illustrated by the solid line for the binding of amaranth in Fig. 1. The agreement with the experimental data is quite satisfactory. Obviously the simplified theoretical approach is adequate for the description of trivalent anions also.

A value of 8×10^4 was found for k_1, the binding constant of the first amaranth complex, PA. For methyl orange and azosulfathiazole values of 4.9×10^4 and 1.25×10^5, respectively, have been obtained previously.[1] The intrinsic binding constants would have the same *relative* values. It is of interest to note that both azosulfathiazole and amaranth exhibit greater intrinsic affinities for the protein than does methyl orange. The stronger attraction must be related to the added van der Waals interactions in the larger molecules. Since azosulfathiazole and amaranth are about the same in size, the weaker affinity of the protein for the latter molecule would seem to be the result of the symmetrical distribution of the charged sulfonate groups. If only one sulfonate is attached to the protein, the other two on the same dye molecule would tend to stay out in the solvent, both because of interaction of the charged groups with the water molecules and because of repulsion by the negatively-charged protein. Since the sulfonates are distributed rather symmetrically, the distance between the protein and hydrophobic portions of the dye also would be increased. In view of the very rapid decrease in the van der Waals interaction with increasing distance, the result of even a small separation between the aromatic rings of amaranth and the protein molecule would be a substantial decrease in the intrinsic stabilization energy of the complex. In contrast azosulfathiazole has both sulfonate groups on one edge of the molecule. Consequently, the long chain aromatic and heterocyclic groups can approach the protein molecule more closely. The proximity of hydrophobic portion of the dye and the protein increases the van der Waals interaction and hence gives a more stable complex.

While no investigations have been made in this Laboratory of the binding of anions with more than three charges, it is evident from the results which have been obtained that further increases in the number of charges would greatly decrease the extent of complex formation, unless the electrostatic repulsion were more than matched by increased van der Waals interactions. Thus it becomes quite clear why Bennhold observed[3] strong binding of coproporphyrins by albumin but no binding of uroporphyrins despite the great similarity in ring structure. For the latter substances have four more carboxyl groups than the former, and since these would be appreciably ionized at plasma pH's, the additional electrostatic repulsion would overcome any tendency toward complex-formation. Similar phenomena must be involved also in the interaction of nucleic acids with serum albumin.[4]

Effect of Changes in pH.—It is generally believed that the foci of attachment of sulfonate and sulfate groups are the positively-charged nitrogen atoms of the protein.[1,2,5,6] These cationic groups must be on the basic amino acids, histidine, lysine and arginine. Since the basic substituents of these amino acids are quite different in chemical nature, their pK's are also separated by rather large intervals (Table I). Investigations of the dependence of binding on pH should lead, therefore, to some indication of the type of quaternary nitrogen atom which has such strong affinity for anions.

It has been shown previously[2] that changes in absorption spectra give quantitative data on the extent of binding of a dye, such as azosulfathia-

(3) B. D. Davis, *Am. Scientist*, **34**, 611 (1946).

(4) E. Stenhagen and T. Teorell, *Trans. Faraday Soc.*, **35**, 743 (1939).

(5) H. P. Lundgren, D. W. Elam and R. A. O'Connell, *J. Biol. Chem.*, **149**, 183 (1943).

(6) F. W. Putnam and H. Neurath, *ibid.*, **159**, 195 (1945).

TABLE I

ACIDITY CONSTANTS OF BASIC GROUPS IN PROTEINS[7]

Amino acid	Basic group	*pK* (25°)
Histidine	Imidazolium	5.6– 7.0
Lysine	ε-Ammonium	9.4–10.6
Arginine	Guanidinium	11.6–12.6

zole, up to concentrations in which an average of 1.5 anions is bound by one protein molecule. This approach has been used, therefore, as a rapid method of investigating the *p*H dependence of the degree of binding. A typical set of results with azosulfathiazole and bovine albumin is illustrated in Fig. 2, in which the relative absorption at a single wave length, 4950 Å., is plotted as a function of *p*H. When buffer alone is present, the absorption is constant from a *p*H near 2 up to about 9. Similar results are obtained over the region of 5 to 9 upon the addition of albumin (*ca.* 0.2%). The relatively large scatter in data in the presence of protein is due to variations in the ratio of dye to protein concentration. It is evident that there is no pronounced change in the extent of binding of azosulfathiazole up to *p*H 9. Unfortunately, investigations could not be carried out with this dye above *p*H 9 because of pronounced changes in spectra even in the absence of albumin. These changes must be due to the ionization of the phenolic hydroxyl group on azosulfathiazole, and since they are far greater than those obtained upon addition of protein, it is difficult to estimate the effect of the latter alone.

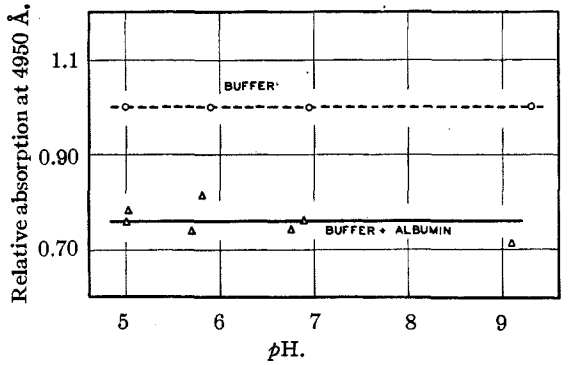


Fig. 2.—Relative absorption of azosulfathiazole at 4950 Å., as a function of *p*H.

To determine the effect of high *p*H on binding it is necessary, therefore, to work with a dye which lacks a phenolic hydroxyl, *e. g.*, methyl orange. The variation of binding with *p*H for methyl orange is summarized in Fig. 3. With this dye, there is no significant change in absorption in buffer solutions from *p*H 5 to almost 13. This constancy of optical behavior is to be expected since none of the substituents loses or gains H^+ ions above about *p*H 4.5. The absorption in the presence of albumin, however, stands in marked contrast to that in buffer alone. While the optical density is essentially constant at *p*H's up to 9, there is a clear break and a trend toward the albumin-free spectrum at higher *p*H's. Above a *p*H of approximately 11.5, there is no significant difference between the spectra in the presence and in the absence of albumin, respectively.

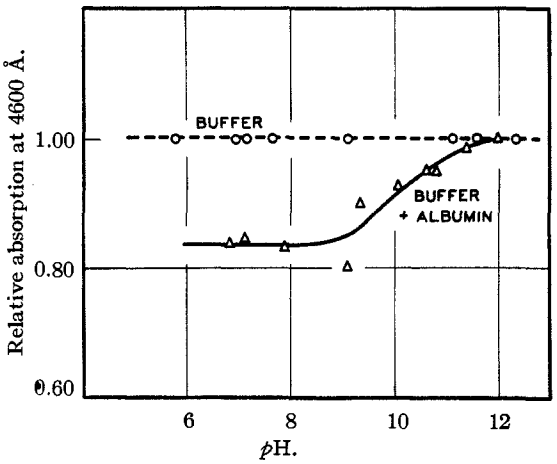


Fig. 3.—Relative absorption of methyl orange at 4600 Å., as a function of *p*H.

In the solutions examined the concentration of methyl orange was approximately 1.2×10^{-5} molar and that of the protein near 0.3%, so that about 80% of the dye would be in the bound form at a *p*H of about 6. From the behavior illustrated in Fig. 3, it seems that the extent of binding is about the same up to *p*H 9. Above this *p*H, however, the degree of complex-formation between dye and protein decreases appreciably and disappears in sufficiently alkaline solution.

On the basis of the experiments described so far, it is conceivable that the decreased binding in alkaline solution is due to alterations in the framework of the protein rather than to changes in H^+ ionization of the basic amino acids. Such structural modifications, however, would presumably be irreversible in character, so that if the protein were returned from *p*H 12 to perhaps 7, it should not regain its binding capacity. The observed behavior is contrary to this prediction. In experiments in which bovine serum albumin was kept at *p*H 12 for periods up to two hours and then acidified and brought to *p*H 7.6, the effect of the protein on the spectrum of methyl orange could not be distinguished from that of a protein solution which had been maintained entirely at *p*H 7.6. The binding capacity was completely regained upon returning the protein from *p*H 12 to 7.6. Thus it seems clearly established that the loss in binding capacity in alkaline solution is due to some *reversible* change in the protein.

As a check on the interpretation of spectral alterations in terms of loss in dye-affinity by the protein, it seemed desirable to make some direct binding measurements. Since the dialysis-equilibrium method requires a period of three days, it

(7) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 445.

seemed best not to use this procedure, for the question of denaturation of the protein in basic solution might be raised. As an alternative a number of ultrafiltration experiments was carried out. Two of these are summarized in Table II. In both cases, the ultrafiltrate in the presence of albumin had practically the same concentration of methyl orange as that obtained from the control tube containing no protein. Clearly no significant binding of methyl orange by bovine serum albumin occurs at *p*H's near 12.

TABLE II

ULTRAFILTRATION OF METHYL ORANGE–ALBUMIN SOLUTIONS

Expt.	*p*H	Conc. of albumin grams/liter	Concn. of M.O. in filtrate
1	11.73	..	2.04×10^{-5}
	11.85	1.90	2.09×10^{-5}
2	11.82	..	5.75×10^{-5}
	11.84	1.89	5.73×10^{-5}

These results indicate that insofar as binding of sulfonated compounds is concerned, the protein undergoes a critical, but reversible, change in the region near *p*H 10. Inspection of Table I reveals that this is also the region in which the ϵ-ammonium group of lysine is losing an H^+ ion, and, therefore, its positive charge. One is thus led to the belief that the stabilization of the protein–dye complex is strongly dependent on the electrostatic interaction with the cationic nitrogen of lysine. It is not clear, however, why lysine should occupy this special position. From the observation[2] that salmine gives a precipitate with either methyl orange or azosulfathiazole, it is evident that the guanidinium group is also capable of strong interaction with sulfonate anions, since this protamine contains over 87% arginine. Consequently, since an appreciable fraction of the guanidinium groups in albumin should still be in the cationic state at a *p*H near 11.5, one would expect some binding of the dye. The absence of spectral alterations at this *p*H must indicate that the arginine residues are in a relatively inaccessible position in the serum albumin molecule or that the guanidinium cations are bound to anionic groups within the protein.

Acknowledgment.—These investigations were supported by grants from the Permanent Science fund of the American Academy of Arts and Sciences and from the Office of Naval Research.

Summary

Investigations have been made of the binding of a triply-charged sulfonated dye (F D and C Red No. 2) by bovine serum albumin. The extent of binding can be correlated in terms of equations derived from the law of mass action.

Spectral methods and ultrafiltration experiments have been used to study the effect of *p*H on protein–anion interactions. Binding phenomena disappear near *p*H 12. Such behavior indicates that the ammonium group of lysine is strongly involved in these interactions.

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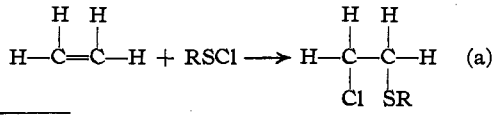
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Derivatives of Sulfenic Acids. I. Reactions of 2-Nitrobenzenesulfenyl Chloride, 2,4-Dinitrobenzenesulfenyl Chloride and of the Corresponding Sulfenyl Thiocyanates with Olefins and with Methyl Ketones

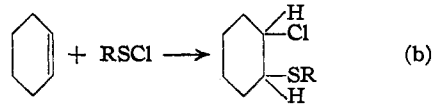
BY NORMAN KHARASCH,[1] HERBERT L. WEHRMEISTER AND HENRY TIGERMAN

We have recently undertaken a series of studies concerning derivatives of sulfenic acids.[2] This paper describes certain new reactions of the 2-nitro- and 2,4-dinitrobenzenesulfenyl chlorides and thiocyanates.

Lecher and Stöcklin[3] reported three instances of the addition of sulfenyl chlorides to ethylene (equation (a), R = phenyl, *p*-tolyl or 2-nitrophenyl).



Fuson, Price and co-workers[3a] similarly added 2-chloroethanesulfenyl chloride to ethylene, cyclohexene and propylene. New examples of this interesting reaction were found in this study. Thus, 2-nitrobenzenesulfenyl chloride and 2,4-dinitrobenzenesulfenyl chloride add to cyclohexene to give corresponding 2-chlorocyclohexyl aryl sulfides (equation (b), R = 2-nitrophenyl or 2,4-dinitrophenyl).



Only single modifications of the latter products were obtained, although geometric isomers are possible.

Reactions analogous to a and b, wherein sul-

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(2) The sulfenic acids are generally designated as RSOH. Known derivatives include the sulfenyl halides (RSX), the sulfenyl thiocyanates (RSSCN), the sulfenamides ($RSNR_2$), the sulfenates (RSOR'), and sulfenic anhydrides (RSOSR). *Cf.*, Kharasch, Potempa and Wehrmeister, *Chem. Rev.*, **39**, 269–332 (1946).

(3) Lecher and Stöcklin, *Ber.*, **58**, 414 (1925).

(3a) Fuson, Price, *et al.*, *J. Org. Chem.*, **11**, 469–481 (1946).