[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Spectrophotometric Investigations of the Interactions of Proteins with Organic Anions

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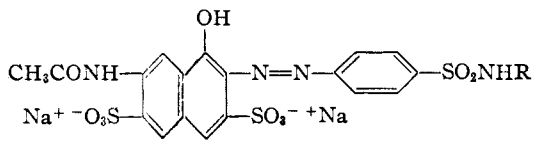
The interactions of organic ions with proteins have been explored by spectral studies[1-10] as well as by other methods of investigation.[11] Spectroscopic experiments, however, have been concentrated largely on solutions of organic cations in the presence of protein anions, or of organic anions in the presence of protein cations. Spectral shifts under these conditions may be attributed to the electrostatic attraction of the oppositely charged entities.[1,5,10] Changes in absorption spectra have been observed also when added proteins disturb the polymer-monomer equilibrium of a dye, even in media where both species have similar charges.[8,10] Few investigations have been made, however, of spectral displacements in solutions of monomeric organic anions and negatively charged proteins. Under these conditions specific attractive forces must exist which overcome the repulsion between ions of similar charge. An understanding of the nature of these forces is essential to the comprehension of many biochemical actions.

Experimental

Reagents.—A sample of azosulfathiazole (I), where R is a thiazole ring, was kindly supplied by the Winthrop Chemical Company.

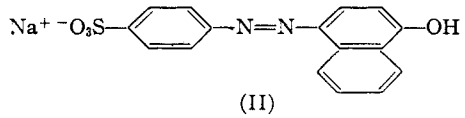


(I)

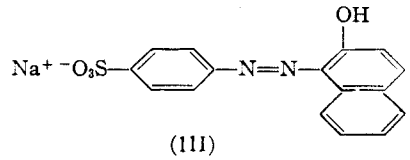
A purity of 97.2% had been found by titration with methylene blue. As indicated by the manufacturer, the impurity is probably only sodium chloride, used in salting out the dye.

Orange I (II), and Orange II (III), were obtained through the courtesy of the National Aniline Division of Allied Chemical and Dye Corporation. The purity of these substances had been determined by titanium chloride titration and was 92.5% for Orange I and 95% for Orange II.

The methyl orange, $Na^+{}^-O_3S—C_6H_4—N=N-C_6H_4—N(CH_3)_2$, was a commercial sample of reagent grade.



(II)



(III)

Crystalline bovine serum albumin and purified bovine γ-globulin were furnished through the courtesy of the Armour Laboratories. The gelatin was Eastman Kodak Co. "Purified Calfskin Gelatin" with an isoelectric point of 4.7 and an ash content of 0.023%.

Sodium dodecyl sulfate was a specially purified sample generously supplied by the Fine Chemicals Division of the du Pont Company. The Carbowax was a commercial sample used without further purification.

The amino acids used were all of reagent grade. The glutathione and salmine were commercial samples and were used without further purification. The organic acids used in the competition experiments were either of "highest purity" grade (Eastman) or were recrystallized twice from appropriate solvents.

Buffer solutions were made from phosphate or borate salts and were approximately 0.1 molar in concentration.

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 and extinction coefficients were calculated from the familiar equation

$$\epsilon = \frac{1}{cd} \log_{10} (I_0/I) \qquad (1)$$

where I_0 is the intensity of the light passing through the solvent, I, the intensity of the light passing through the solution, c, the molar concentration of solute, and d, the thickness of the absorption cell in centimeters.

Results and Discussion

Spectral Changes and Complex-formation.—The spectral properties of azosulfathiazole are typical of a large number of dyes which have been investigated. Its absorption spectrum in a buffered aqueous solution at a *p*H of 6.92 is illustrated in Fig. 1, and shows a maximum typical of azo compounds at 4950 Å. The magnitude of absorption of light by this substance has been found to be directly proportional to its concentration up to the region of 10^{-3} molar. This adherence to Beer's law indicates that the dye exists in the monomeric state in aqueous solutions below 10^{-3} molar.

The spectrum of this azo compound is not affected significantly by the presence of sodium chloride, at least up to concentrations of 0.5 molar. Similarly the absorption is independent of *p*H over the region of 2 to 9 though there is a pronounced change at higher *p*H's presumably due to the ionization of the phenolic hydroxyl group.

In a solution containing a few hundredths of a

(1) G. S. Hartley, *Trans. Faraday Soc.*, **30**, 444 (1934).

(2) M. I. Gregersen and J. G. Gibson, *Am. J. Physiol.*, **120**, 494 (1937).

(3) D. Keilin and E. F. Hartree, *Nature*, **145**, 934 (1940).

(4) H. W. Robinson and C. G. Hogden, *J. Biol. Chem.*, **137**, 239 (1941).

(5) S. E. Sheppard, R. C. Houck and C. Dittmar, *J. Phys. Chem.*, **46**, 158 (1942).

(6) R. A. Rawson, *Am. J. Physiol.*, **138**, 708 (1943).

(7) P. Dow, *Federation Proc.*, **4**, 16 (1945).

(8) S. E. Sheppard and A. L. Geddes, *J. Chem. Phys.*, **13**, 63 (1945).

(9) T. K. With, *Acta Physiol. Scand.*, **10**, 172 (1945).

(10) L. Michaelis and S. Granick, THIS JOURNAL, **67**, 1212 (1945).

(11) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946). Numerous references to the work of others are given in this article.

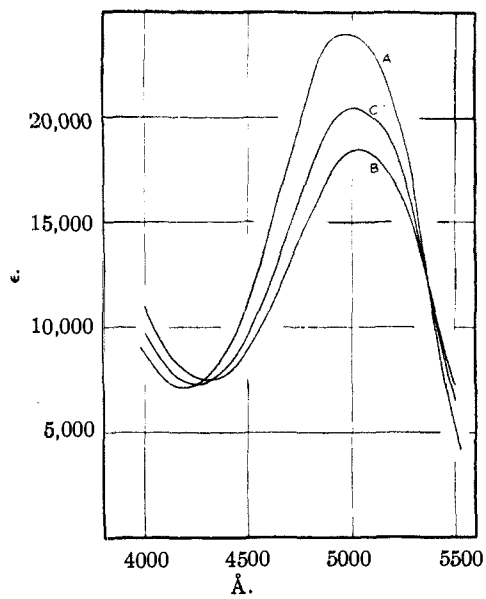


Fig. 1.—Absorption spectra of azosulfathiazole; A, in buffer at *p*H 6.92; B, in buffer containing bovine albumin (0.2%), *p*H 6.90; C, in buffer containing bovine albumin and *p*-aminobenzoic acid (1.34×10^{-2} *M*), *p*H 6.45. The slight difference in height of the maximum in this figure as contrasted to that in an earlier publication [*Science*, **104**, 264 (1946)] is due to the absence of information on the water content of the compound at the time the latter paper was submitted.

per cent. of bovine serum albumin, the spectrum of azosulfathiazole is significantly altered, and very large changes are observed in the presence of 0.2% of the protein (Fig. 1). The observed shifts cannot be due to changes in the *p*H of the medium, for the addition of protein to the aqueous buffer solution decreases the *p*H by a few tenths of a unit and never takes the solution outside the range in which the pure dye shows a constant spectrum. Similarly the alteration of the spectrum cannot be attributed to displacements in dimer or polymer equilibria, for the dye has been shown to be monomeric in aqueous solution. Thus it becomes apparent that the interaction of the bovine albumin and azosulfathiazole is a specific effect due to the combination of the protein with the dye anion to form an intermolecular complex.

The existence of such a complex has been confirmed by direct binding studies,[11] and is also quite evident from the dependence of the spectrum on the concentration of protein. As one would expect from equilibrium principles the addition of protein to a solution with a fixed dye concentration should increase the extent of complex formation. After a certain point, however, further increases should produce no observable effect for practically all of the anion would be in the complex. These predictions are in agreement with the observation that in dye solutions of approximately 1×10^{-5} molar concentration, the addition of albumin up to about 0.2% concentration produces a progressive lowering of the absorption spectra, but further increases up to concentrations as high as 1% produce no additional effect.

This relationship between spectral changes and complex-formation may be substantiated further by comparison of quantitative calculations of the extent of combination from spectroscopic data with the results obtained in direct binding studies.[11] In solutions of low protein concentration which contain both bound and unbound azosulfathiazole, the absorption of light may be expressed by the relation

$$\log (I_0/I) = \epsilon_1 c_1 d + \epsilon_2 c_2 d \qquad (2)$$

where the subscript 1 refers to the unbound anion and the subscript 2 to the bound azosulfathiazole. If we define $\epsilon_{\text{apparent}}$ by the relation

$$\epsilon_{\text{apparent}} = \frac{\log (I_0/I)}{(c_1 + c_2)d} \qquad (3)$$

then it can be shown readily that

$$\epsilon_{\text{apparent}} = \alpha_1(\epsilon_1 - \epsilon_2) + \epsilon_2 \qquad (4)$$

where α_1 is the fraction of dye which is free, and consequently that

$$\alpha_1 = \frac{\epsilon_{\text{apparent}} - \epsilon_2}{\epsilon_1 - \epsilon_2} \qquad (5)$$

Knowing α_1 and the total concentration of dye, one may determine the concentrations of bound and unbound dye by the steps outlined in Table I.

The variation of bound dye with the concentration of free azosulfathiazole is illustrated in Fig. 2. Also plotted in this figure are values determined directly by dialysis-equilibrium experiments.[11] In the region in which the two types of experiment overlap, the agreement is excellent. It is also obvious from the "spectroscopic titration" alone that more than one mole of dye is bound by each mole of protein, but the precision of this method

TABLE I

CALCULATION OF BOUND AZOSULFATHIAZOLE

Concn. of azosulfathiazole, moles/liter	Concn. of albumin moles[a]/liter	$\epsilon_{\text{apparent}}$ at 4950Å.	α_1[b]	Concn. of free azosulfathiazole	Concn. of bound azosulfathiazole	Moles bound azo / Moles total protein
1.59×10^{-5}	2.47×10^{-5}	18000	0.11	0.18×10^{-5}	1.41×10^{-5}	0.57
2.38	2.36	18200	.14	0.33	2.05	0.87
3.97	2.14	19300	.31	1.23	2.74	1.28
0.794	0.515	20400	.49	0.39	0.40	0.78
1.59	0.493	21200	.61	0.97	0.62	1.26

[a] Calculated on basis of 70,000 for molecular weight of bovine serum albumin. [b] In this series of experiments, ϵ_1 = 23,650 and ϵ_2 = 17,300.

falls off rapidly at high concentrations of free dye and hence cannot be used by itself to investigate regions of higher binding,

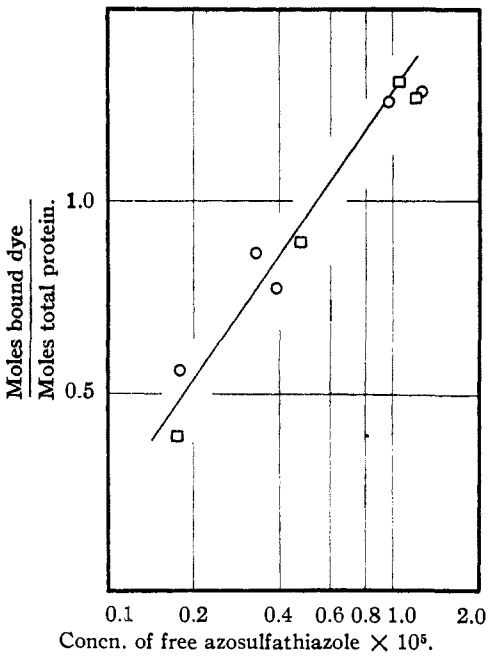


Fig. 2.—Quantitative data on extent of binding: O, from spectral measurement; □, from dialysis-equilibrium studies.

Complex-formation and Nature of Dye.—The formation of a complex with bovine serum albumin is exhibited by compounds other than azosulfathiazole also. Neoprontosil (I, R = H), for example, closely related to azosulfathiazole (I), undergoes the same type of spectral depression in the presence of the protein. Similar behavior is observed with Orange I (II), Orange II (III) and methyl orange, typical curves for which are shown in Figs. 3, 4 and 5. It seems most likely that the

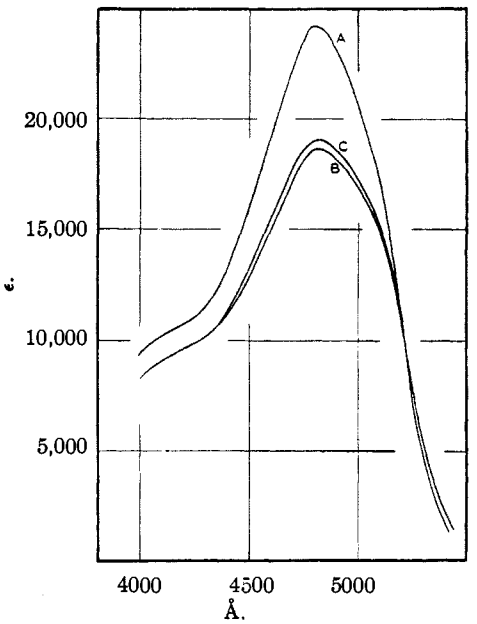


Fig. 4.—Absorption spectra of Orange II: A, in buffer at *p*H 6.83; B, in buffer containing bovine albumin (0.2%), *p*H 6.83; C, in buffer containing bovine albumin and potassium acid phthalate (0.99 × 10^{-2} *M*), *p*H 6.66.

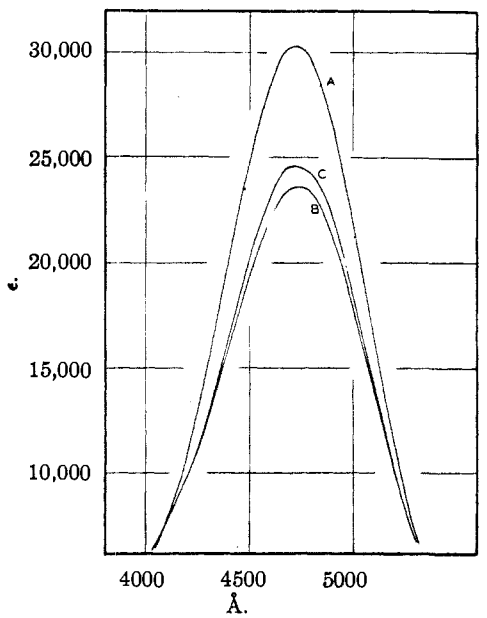


Fig. 3.—Absorption spectra of Orange I: A, in buffer at *p*H 6.81; B, in buffer containing bovine albumin (0.2%), *p*H 6.81; C, in buffer containing bovine albumin and potassium acid phthalate (0.98 × 10^{-2} *M*), *p*H 6.63.

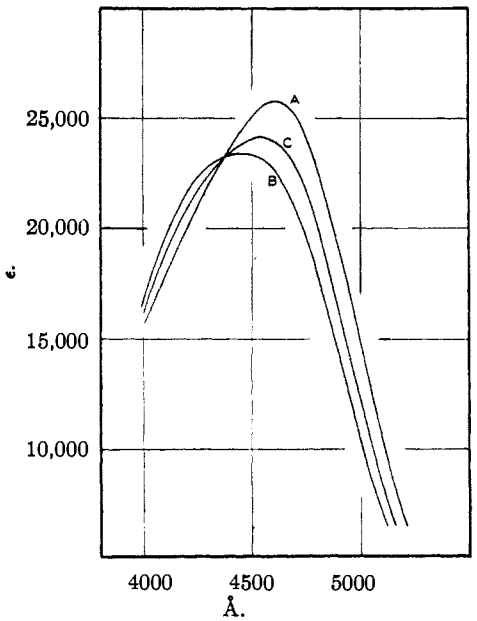


Fig. 5.—Absorption spectra of methyl orange: A, in buffer at *p*H 6.84; B, in buffer containing bovine albumin (0.2%), *p*H 6.83; C, in buffer containing bovine albumin and potassium acid phthalate (0.98 × 10^{-2} *M*), *p*H 6.64.

sulfonate group, common to all of these substances, is primarily responsible for the binding to the protein, as is the case in the interactions of detergents with albumin.[12,13]

While the addition of bovine albumin lowers the value of the maximum extinction coefficient in each dye investigated, the shift in the wave length of the absorption peak is not the same in each case (Table II). Thus the complex with azosulfathiazole has its peak shifted to longer wave lengths, that with methyl orange to shorter wave lengths, while that with Orange I or Orange II, respectively, shows no significant displacement. Apparently the relative effect of the protein on the energies of the ground and excited states of the dyes is dependent on the nature of the entire molecule, and not only on the character of the binding sulfonate group.

TABLE II

EFFECT OF BOVINE SERUM ALBUMIN ON THE POSITION OF THE ABSORPTION MAXIMUM

Dye	Absorption maximum in buffer, Å.	Absorption maximum in presence of protein, Å.
Azosulfathiazole	4950	5050
Orange I	4730	4730
Orange II	4810	4810
Methyl Orange	4600	4400

Complex-formation and Nature of Protein.—The available evidence indicates that quaternary nitrogens on the albumin molecule are the foci of attachment of the organic sulfonate anions. That the changes in absorption are not due merely to van der Waals interactions between the dye anion and the large protein molecule is indicated by the absence of any spectral shifts for azosulfathiazole in the presence of sodium dodecyl sulfate (with a molecular weight of 20,000 in the micellar state in aqueous solution[13]) or on the addition of Carbowax (a commercial polyoxymethylene polymer with a molecular weight of about 6000[14]). It is also of interest to note that no spectral shift is obtained when gelatin is used as the added protein in place of bovine serum albumin. While gelatin is not a homogeneous protein, carefully treated samples have average molecular weights of 60,000[15] or more, values which are quite close to that of serum albumin. Nevertheless no spectral alterations were observed with either methyl orange or azosulfathiazole in solutions containing as high as 1% gelatin, a quantity which is almost five times as much as is necessary to produce a maximum effect with bovine albumin.

The spectra of methyl orange in solutions of bovine γ-globulin at a concentration of 0.2% and at *p*H's of 6.9 and 7.4 were examined also, but no significant protein effect was observed. Such behavior is in agreement with the observation from dialysis studies that γ-globulin does not bind methyl orange.[16]

With 0.2% bovine serum albumin, the spectral depression for either methyl orange or azosulfathiazole has been found to be of about equal magnitude at all *p*H's from 5 to 9. This would indicate that the quaternary nitrogens involved in this interaction are those of the ϵ-amino group of lysine or of the guanidinium group of arginine in the protein, for histidine has a pK in the region of 5 to 9 and one would expect a decreased interaction on the basic side of its ionization constant. On the other hand, lysine and arginine with pK's between 10 to 12[17] would exist overwhelmingly in the positively charged quaternary form at all *p*H's below 9 and hence would interact electrostatically with the negative sulfonate ion.

The addition of individual amino acids (0.2% concentration) at *p*H's near 7, to solutions of either azosulfathiazole or methyl orange produces no spectral shift. The basic amino acids, arginine, histidine and lysine, as well as others such as glycine, tyrosine and cysteine were investigated. Negative results were obtained also with glutathione. On the other hand the addition of either methyl orange or azosulfathiazole to an aqueous solution of salmine produces a precipitate. Since this protamine contains over 87% arginine,[18] its precipitation by these dyes indicates strong interaction with the sulfonate anion. The absence of interactions with the free amino acids indicates that the electrostatic attraction must be supplemented by van der Waals forces in order that a stable bond may be formed. A method of evaluating the magnitude of these forces has been outlined in a previous paper.[11]

Competition Phenomena.—The addition of any one of a large number of organic acids to a solution of the protein–dye complex will reverse the effect of the protein on the spectrum of the azo compound. This reversal has been observed with each of the dyes investigated and typical examples are illustrated in Figs. 1, 3, 4 and 5. The extent of reversal depends on the concentration of the added organic acid. Apparently the latter compound displaces the azo dye from the protein complex. The carboxyl ion and the sulfonate ion thus must compete for the points of attachment on the protein molecule.

It is of interest to compare the extent of reversal for the three "orange" compounds which are very similar in that each contains but one sulfonate group. A comparison of the corresponding spectra in the presence of potassium biphthalate indicates that methyl orange is more readily displaced from the protein than is either Orange I

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

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

(14) C. P. McClelland and R. L. Bateman, *Chem. Eng. News*, **23**, 247 (1945).

(15) N. R. Joseph, *J. Biol. Chem.*, **126**, 389 (1938).

(16) Unpublished work in this Laboratory.

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

(18) E. J. Cohn and J. T. Edsall, *ibid.*, p. 358.

or Orange II. The most significant difference between these latter two compounds and methyl orange is the presence of an additional aromatic ring in Orange I and Orange II. This added ring apparently contributes an appreciable portion to the binding energy of the protein-dye complex. This effect is also apparent from the observation that concentrations of carboxyl compound of the order of 10^{-2} molar will displace only a fraction of the dye from the complex even though the concentration of azo compound is only about 10^{-5} molar. That this weaker affinity of the carboxyl compound is not due to the difference in relative attraction to the protein of the carboxyl and sulfonate groups will be evident from the discussion to follow. Obviously then, the greater strength of attachment of the azo compounds to the protein must be due to the additional van der Waals forces in these large molecules.

It is also of interest to note that Orange I is more readily displaced from its complex than is Orange II or, in other words, that the latter is more strongly bound by the protein. The only difference between these two dyes is in the position of the phenolic hydroxyl group. The greater affinity of Orange II, with the hydroxyl group ortho to the azo group, may lie in the fact that an intramolecular hydrogen bond is formed in this compound, so that the —OH group does not interact strongly with the solvent water molecules. On the other hand, in Orange I, the hydroxyl group is in the para position and hence cannot form an intramolecular hydrogen bond. In consequence, the para —OH group can form hydrogen bonds readily with the water molecules, and hence Orange I would be subject to an attraction by the solvent which would be greater than that of Orange II and which would make its protein affinity relatively weaker than that of Orange II. Such an interpretation is in line with the observation that salt formation is inhibited in dyes containing a phenolic hydroxyl ortho to an azo group.[19]

The degree of displacement of methyl orange or azosulfathiazole, respectively, by a given carboxylic acid seems to be approximately the same under equivalent concentration and *p*H conditions. Binding studies[11] indicate that the maximum number of bound molecules of azosulfathiazole is the same as that for methyl orange. This fact would mean that only one sulfonate group on each azosulfathiazole molecule is involved in the complex with the protein and that the second one is relatively free for interaction with solvent water molecules. The interactions of this second sulfonate group, as well as the other polar substituents on azosulfathiazole, with the solvent molecules would tend to decrease the affinity of the dye for the protein and hence compensate for the increase in affinity promoted by the van der Waals attraction of the additional aromatic ring. The net result of these counteracting effects is a complex with a stability roughly the same as that of methyl orange.

A fairly extensive survey of the competitive abilities of a number of different organic acids has been made for methyl orange and azosulfathiazole, respectively. The results obtained are summarized in Tables III and IV. The reversing effect has been expressed in terms of $\Delta\epsilon$, the increase in extinction coefficient of the dye in an albumin solution containing the organic acid over that observed with only albumin and dye. This method of expression has been adopted in preference to mere listing of the absolute value of the extinction coefficient in albumin-organic acid solutions, because the absolute value in such a solution, as well as in one containing albumin but no carboxyl compound, is affected by small variations in concentration of dye, corresponding to slight changes in the extent of binding.

TABLE III

COMPETITION BETWEEN CARBOXYLIC ACIDS AND METHYL ORANGE

Compound	Concn., moles/liter	*p*H	$\Delta\epsilon$ at 4600 Å.	% Compd. in form of anion[a]
Salicylic acid	1.46×10^{-2}	6.88	3100	100[d]
Potassium acid phthalate	1.01×10^{-2}	5.26	2600	$50A^-$, $50A^{=}$[d,e]
	0.98×10^{-2}	6.64	1200	$4A^-$, $96A^{=}$[d,e]
Benzoic acid	1.64×10^{-2}	7.04	2200	100[f]
p-Chlorobenzoic acid	1.02×10^{-2}	6.52	2100	100[f]
o-Chlorobenzoic acid	1.02×10^{-2}	6.52	2000	100[f]
o-Aminobenzoic acid	1.46×10^{-2}	6.56	2200	98[b]
p-Aminobenzoic acid	1.45×10^{-2}	6.88	1100	99[b]
Sulfanilic acid	1.15×10^{-2}	6.61	1400	100[b]
Maleic acid	1.73×10^{-2}	6.67	1100	$20A^-$, $80A^{=}$[c]

[a] These calculations are based on ionization constants obtained from the following sources: [b] "Landolt-Börnstein, Physikalisch-chemische Tabellen," 5th edition; [c] *ibid.*, Erg. I; [d] *ibid.*, Erg. II; [e] *ibid.*, Erg. III; [f] H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, N. Y., 1943, p. 210; [g] P. H. Bell and R. O. Roblin, Jr., THIS JOURNAL, **64**, 2905 (1942); [h] M. E. Cupery, *Ind. Eng. Chem.*, **30**, 627 (1938).

TABLE IV

COMPETITION BETWEEN CARBOXYLIC ACIDS AND AZOSULFATHIAZOLE

Compound	Concn., moles/liter	*p*H	$\Delta\epsilon$ at maxima	% Compd. in form of anion[a]
p-Chlorobenzoic acid	1.18×10^{-2}	6.51	5100	100[f]
o-Chlorobenzoic acid	1.18×10^{-2}	6.51	4500	100[f]
o-Aminobenzoic acid	1.35×10^{-2}	6.45	4500	97[b]
p-Aminobenzoic acid	1.34×10^{-2}	6.45	2000	97[b]
p-Aminobenzoic acid	1.44×10^{-2}	9.02	0	100[b]
Sulfanilic acid	0.96×10^{-2}	6.55	1400	100[b]
Sulfanilamide	1.31×10^{-2}	5.12	0	0[g]
Sulfamic acid	2.06×10^{-2}	8.81	0	100[h]
Salicylic acid	1.47×10^{-2}	8.98	3100	100[d]
Potassium acid phthalate	1.98×10^{-2}	4.79	4300	$75A^-$, $24A^{=}$[d,e]
	0.49×10^{-2}	5.42	3700	$41A^-$, $59A^{=}$[d,e]
	0.38×10^{-2}	9.02	400	$100A^{-}$[d,e]
Maleic acid	1.60×10^{-2}	5.82	3000	$64A^-$, $36A^{=}$[e]
Maleic acid	1.65×10^{-2}	8.64	200	$100A^{=}$[e]
Succinic acid	1.71×10^{-2}	4.38	0	$58A^-$, $5A^{=}$[e]
Succinic acid	1.65×10^{-2}	8.72	0	$100A^{=}$[e]
Veronal	1.12×10^{-2}	5.12	0	0[c]

[a] These calculations are based on ionization constants obtained from the sources indicated in the footnotes to Table III.

(19) W. B. Reynolds, *Chem. Eng. News*, **24**, 916 (1946).

In all cases investigated, the reversing ability of a given carboxylic acid decreases with increase in *p*H. This trend is quite clear from the data on potassium biphthalate in Tables III and IV as well as from those on *p*-aminobenzoic acid and maleic acid in Table IV. The decrease in affinity at high *p*H must be an expression of the increasing electrostatic repulsion of the organic anion due to the increasing negative charge on the protein molecule. The importance of this repulsion in determining the relative attraction of two organic anions to a protein molecule will depend on the contributions of electrostatic and van der Waals factors to the stability of the complex. Van der Waals forces would contribute more to the stability of the protein-azo complexes than to that of the protein–organic anion complexes, since the latter have at most but one aromatic ring. Thus, at high *p*H's, electrostatic repulsion is relatively more effective against the organic anions than against the azo compounds, and hence the competing ability of the former decreases. On the other hand, in competitions between molecules which are more nearly alike in structure, as, *e.g.*, *p*-aminobenzoic acid and a simple sulfonamide anion, electrostatic repulsion should be of approximately equal importance in both cases.

As has been implied above, the data in Tables III and IV indicate that the sulfonate and carboxyl groups are not greatly different in their affinity for the protein. A comparison of the $\Delta\epsilon$'s (at approximately the same *p*H) for *p*-aminobenzoic acid and sulfanilic acid, completely analogous except for the replacement of the carboxyl group by a sulfonate group, shows practically the same reversing ability. The small differences are probably due to the slight variations in *p*H and concentration between the different experiments. In contrast there are very large differences between two isomers, such as *o*- and *p*-aminobenzoic acid. *o*-Aminobenzoic acid is approximately twice as effective as the para isomer both against methyl orange and azosulfathiazole. Similarly two other ortho-substituted compounds, potassium acid phthalate and salicylic acid are very effective reversing agents. However, ortho substitution is not a necessary condition for strong affinity for the protein, for *p*-chlorobenzoic acid is a very effective reversing agent against azosulfathiazole or methyl orange, and benzoic acid is capable of displacing a large fraction of methyl orange from its protein complex. Furthermore, maleic acid, which is not even aromatic in character, is an effective reversing agent against both of the dyes tested. Succinic acid, however, shows little activity, but its ineffectiveness may lie in the fact that it is not highly ionized at *p*H 4.38, and is merely exhibiting the general unfavorable effect of high *p*H at 8.72. The inability of sulfamic acid to cause reversal may also be due to the high *p*H. The ineffectiveness of veronal and sulfanilamide, on the other hand, is probably to be attributed to the absence of the anionic forms at the *p*H which was used.

Thus it seems that only the anionic form of the organic acid is capable of displacing the azo dye. It must be kept in mind, however, that this spectrophotometric method is not capable of distinguishing between small differences in binding ability and hence a small reversing ability of the neutral acid may not show up. Nevertheless, it is quite clear that the anion is much more effective than the acid molecule as one might expect if electrostatic attraction is an appreciable fraction of the binding force. Within the list of anions there are also some striking differences in reversing ability, as one can see by comparison of salicylic acid and potassium acid phthalate, or *o*- and *p*-aminobenzoic acid. The basis of these differences probably lies in differences in ability to form hydrogen bonds as well as in configurational patterns of the protein, but a clear understanding of these variations must await further study.

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Summary

The spectra of a number of azo dyes containing sulfonate groups undergo pronounced alterations in the presence of low concentrations (0.1%) of bovine serum albumin. γ-Globulin and gelatin, as well as certain polymeric materials, produce no such change. The spectral shifts are due to complex-formation between the dye and the protein. Quantitative calculations of the extent of this complex-formation have been made from the spectral studies and compared with direct determinations from dialysis–equilibrium measurements.

The stability of the dye–protein complex is determined both by electrostatic forces between the sulfonate ion and a quaternary nitrogen on the protein and by van der Waals interactions, particularly with aromatic rings in the dye. The azo compound may be displaced from the complex by the addition of any one of a number of carboxylic acids, particularly those of aromatic nature. Only the carboxylate anion seems capable of competing with the sulfonate dye, and in all cases examined the competitive ability of the organic acid decreases with increase in *p*H. No clear relation is evident yet between structure and displacing ability of an organic acid.

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