THE EFFECTS OF SALTS AND PROTEINS ON THE SPECTRA OF SOME DYES AND INDICATORS[1,2]

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I. INTRODUCTION

Environmental changes frequently produce alterations in spectra which are even more profound than those obtained from modifications in molecular structure. The effects of changes in solvent or physical state have been investigated widely, and much of the pertinent information has been correlated in a review by Sheppard (18). In connection with biochemical studies, an investigation of the effects of salts and proteins on dye spectra are of primary interest, not only because they point out the limitation of colorimetric methods in determining the pH inside microscopic cells, but also because they give an insight into the nature of the interactions between electrolytes, and between proteins and small molecules.

The effects of both salts and proteins can be explained largely on the basis of established thermodynamic principles applicable to ionic equilibria. Some recourse must be made, however, to specific interactions, the nature of which is not yet entirely clear.

[1] Presented at the Symposium on Color and the Electronic Structure of Complex Molecules which was held under the auspices of the Division of Physical and Inorganic Chemistry of the American Chemical Society at Northwestern University, Evanston and Chicago, Illinois, December 30 and 31, 1946.

[2] Most of the work on salt effects was carried out in coöperation with Dr. C. R. Singleterry under the direction of Professor T. F. Young at the University of Chicago.

II. SALT EFFECTS

It has long been realized that colorimetric methods give erroneous pH values in solutions of high salt content (23). Such errors can be traced back fundamentally to spectral shifts upon addition of the electrolyte. A typical example of such a shift is illustrated for the indicator methyl orange in figure 1. Extensive studies of these shifts under a wide variety of conditions have shown that these "salt errors" may be attributed to one or more of the following factors: (*1*) changes in the absorption spectrum of one or both forms of the indicator dye;

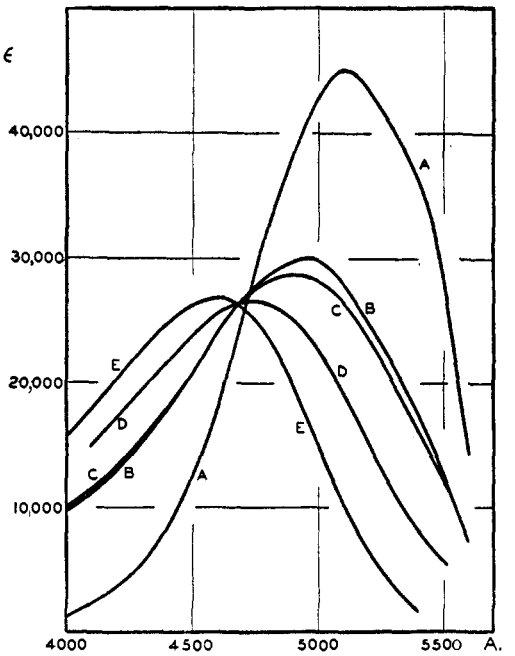


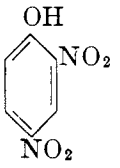
FIG. 1. Salt effects on spectra of methyl orange. A, indicator in solution of hydrochloric acid, pH 1.98; B, indicator in solution of hydrochloric acid, pH 3.51; C, indicator in solution B with addition of potassium chloride to give 0.25 molar solution; D, indicator in solution B with addition of sodium sulfate to give 0.06 molar solution; E, indicator in phosphate buffer, pH 7.73.

(*2*) a shift in equilibrium between acid and base forms of the indicator due to electrostatic interactions; (*3*) changes in pH of the medium due to removal of some hydrogen ion by combination with the salt. The last effect is quite pronounced even with salts, such as sodium sulfate, which generally are not considered to change the pH appreciably. Careful investigations of all of these effects have given not only information on the properties of the dyes but also a much clearer insight into the nature of ionic interactions in aqueous solutions.

A. Changes in spectra of end forms of indicator dye

The most precise optical investigations of the effect of a salt on the spectrum of an end form of an indicator are those of von Halban and Kortüm (8, 25).

Having at their disposal a differential spectrophotometer capable of a precision of better than 0.01 per cent, these investigators studied the effects of numerous salts in exceedingly dilute solution (*ca.* 10^{-4} molar). The dependence of spectral shifts of the dinitrophenolate ion on the charge type of the added electrolyte



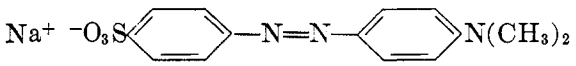
I

2,4-Dinitrophenol

is illustrated in figure 2. The very large increase in absorption with di- and trivalent ions is particularly striking. It is also of interest to note that the uni-univalent salts affect the absorption in a manner opposite to that observed with the polyvalent ions. Spectral shifts are observed also in dilute solution of added non-electrolyte such as urea.

There seems to be no relation between the magnitude of the spectral shift and the activity coefficient of the added ion. Consequently, the optical changes in the dinitrophenolate ion cannot be attributed to ion–cloud interactions of the Debye–Hückel type (8). An explanation in terms of ion association also seems unlikely, because anions are capable of causing deviations equal in magnitude to those observed with cations. Optical variations seem to be determined rather by interactions between added ions and the solvent molecules surrounding the light-absorbing ion (8). Such a conclusion is substantiated by the correlation between the magnitude of shift produced by a given ion and the value of its hydration energy.

An ion such as that of 2,4-dinitrophenol would be particularly sensitive to electrolyte–solvent interactions, since the dye has three highly exposed polar groups which must interact strongly with the solvent molecules and which simultaneously are intimately involved in the resonance which determines the stability of the ground and excited states of the molecule. Somewhat smaller effects might be expected for the end forms of methyl orange (II) with fewer highly



II

Methyl orange

polar substituents. Figure 3 summarizes some of the effects observed with methyl orange by Sidgwick, Worboys, and Woodward (20). The deviations are somewhat less than those for sodium perchlorate on dinitrophenol and specific anion effects are not observable.

B. Effects on activity coefficients of indicators

From figure 3 it is obvious that the large salt deviations illustrated in figure 1 cannot be attributed to changes in the spectra of the end forms of methyl orange,

for such changes are negligibly small at concentrations of electrolyte near 0.1 molar. The effect of a completely unhydrolyzed salt, such as potassium chloride, must be ascribed, therefore, to electrostatic interactions which alter the equilibrium between acid and base forms of the indicator. Such an explanation in terms of ion-cloud interactions can account for the magnitude of the deviations observed, and in the limit of the infinitely dilute solutions, leads to excellent agree-

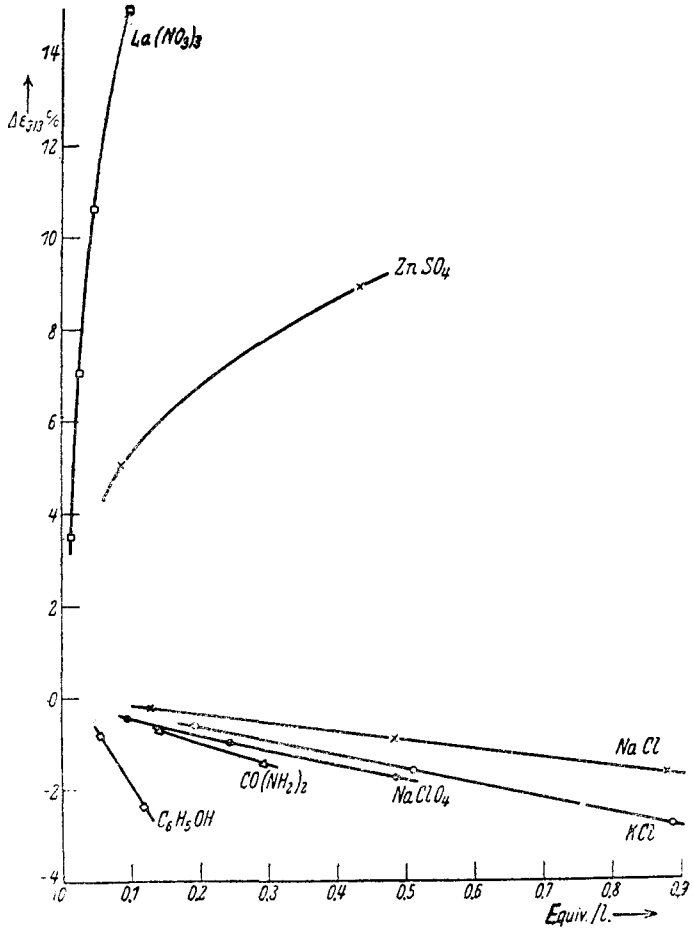


FIG. 2. Effect of various substances on the optical absorption of the 2,4-dinitrophenolate ion.

ment between observed behavior and that predicted by the Debye-Hückel theory.

Since the behavior observed with methyl orange is typical of a large number of indicators, we may generalize our analysis by the following notation: If we allow the acid form of the indicator to be represented by HI, then the dissociation equilibrium may be described by the equation:

$$HI = H^+ + I^- \qquad (1)$$

The corresponding equilibrium constant, K, would be expressed by the relation

$$\frac{(H^+)(I^-)}{(HI)} \frac{\gamma_{H^+}\gamma_{I^-}}{\gamma_{HI}} = K \tag{2}$$

where the parentheses represent molar concentrations and γ's the activity coefficients. Conversion of equation 2 to the logarithmic form leads to:

$$\log \frac{(I^-)}{(HI)} = \log K - \log (H^+) - \log \frac{\gamma_{H^+}\gamma_{I^-}}{\gamma_{HI}} \tag{3}$$

The spectrum, or color, of the indicator solution is determined by the ratio $(I^-)/(HI)$. From equation 3 it is clear that this ratio can be affected only by a

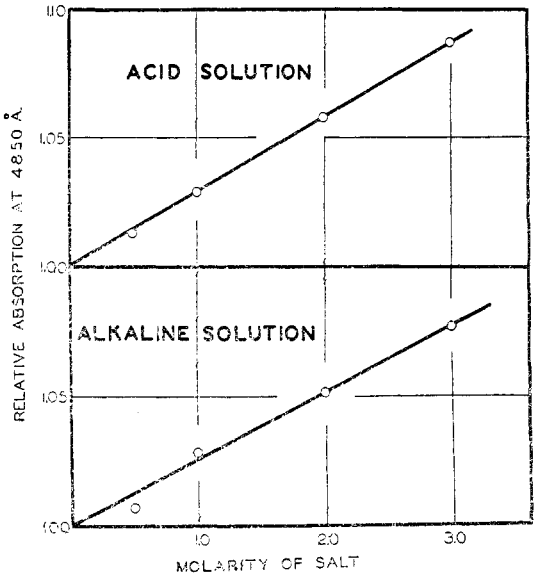


FIG. 3. Effect of salts (sodium chloride, sodium bromide, sodium chlorate, sodium nitrate, potassium chloride, potassium bromide) on light absorption of end forms of methyl orange (20).

change in (H^+) or in the activity coefficient ratio. Since a salt such as sodium chloride or potassium chloride does not change the hydrogen-ion concentration, the addition of an electrolyte of this type to the indicator solution affects only the activity ratio. From variations of color, and hence of $(I^-)/(HI)$, it is possible, therefore, to evaluate the activity-coefficient ratio at various ionic strengths. A typical set of such investigations for methyl orange (4, 21) is illustrated in figure 4. It is interesting to observe that the activity coefficients are practically the same in solutions of barium chloride as of sodium chloride, despite the change in charge type. Apparently this manifestation of specific ion interactions is also rather small in methyl orange. For both of these salts, the activity-coefficient ratio increases (in absolute magnitude) with increasing temperature, a

behavior characteristic of electrolytes. The shapes of the curves also indicate an increase in the magnitude of the limiting slope, in agreement with the Debye–Hückel theory. At 25°C. a least-squares equation through the sodium chloride data yields, upon differentiation, a limiting slope of -1.09, a value which is in satisfactory agreement with the theoretical one of -1.02. Since the method is capable of giving the limiting slope only within about 10 per cent, it is impossible to make any quantitative comparison with the theoretical prediction of a 9 per cent change between 5° and 55°C., but an inspection of figure 4 indicates good qualitative agreement.

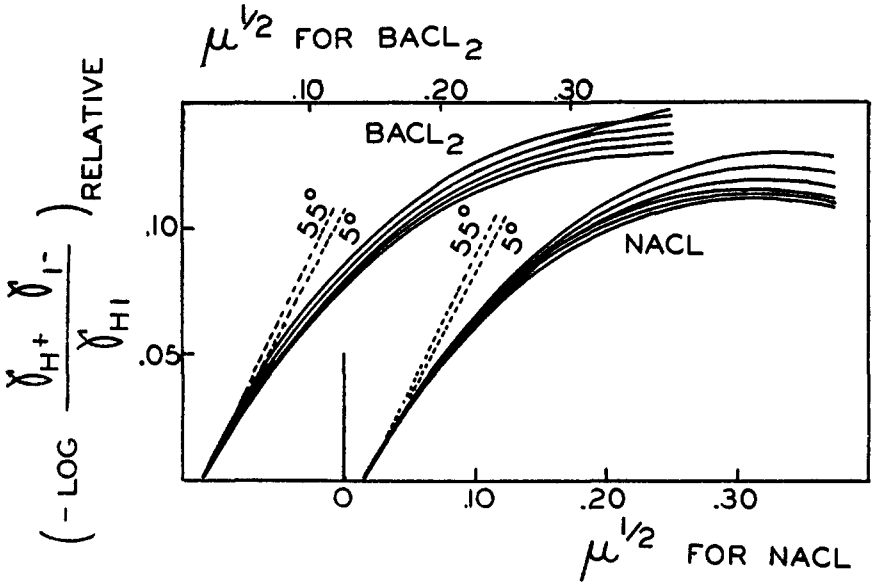
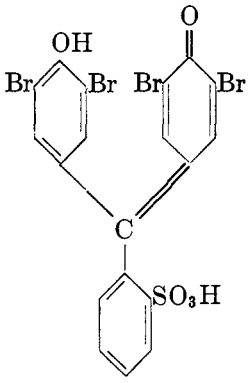


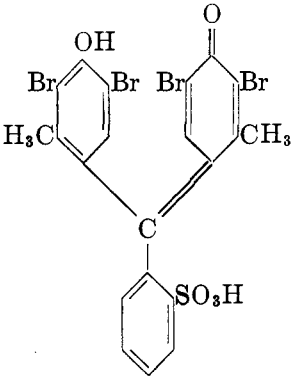
FIG. 4. Activity coefficient ratio (relative to reference solution) for methyl orange in solutions of sodium chloride and barium chloride, respectively. In each case the lowest curve is for 4.7°C., the others at higher temperatures, in intervals of 10°. The dotted lines represent the Debye–Hückel limiting slopes at the temperatures indicated (4, 21).

C. Removal of hydrogen ions by salts of moderately strong acids

It has been realized for some time, of course, that color changes may occur in an indicator solution upon the addition of a salt of a weak acid, e.g., sodium acetate, since acetate ions remove some hydrogen ions. As is obvious from equation 3, the ratio $(I^-)/(HI)$ is affected by changes in pH even if the activity ratio is held constant. That such pH changes might be detectable in dilute acid solutions upon the addition of sodium sulfate, a salt of a moderately strong acid, HSO_4^- ion, was not evident until some observations made by Mullane (12). He found that the addition of alkali metal sulfates to solutions of the indicators bromophenol blue (III) or bromocresol green (IV) produces changes in color which are far greater than the consistently uniform results obtained with alkali



III
Bromophenol blue



IV
Bromocresol green

metal chlorides, bromides, or nitrates. Bromophenol blue and bromocresol green, however, show large neutral salt errors, because the equilibria between acid and base forms involve ions of single and double charge, and hence are not suitable for evaluating the ionization constant of HSO_4^- ion from pH deviations. On the other hand, the neutral salt effects with methyl orange are relatively small (figure 4); hence the large pH deviations upon the addition of sulfates (figure 5) offer a convenient method of determining the ionization constant of a moderately strong acid such as the bisulfate ion.

The equilibrium constants for the reaction

$$HSO_4^- = H^+ + SO_4^{--} \qquad (4)$$

calculated (4, 21) from the data summarized in figure 5 are listed in table 1, together with the values of related thermodynamic quantities. For the dissociation in question the constants obtained are probably the most accurate available. Earlier electromotive-force investigations (3) of the bisulfate dissociation gave results which must certainly be erroneous, since they lead to a heat of dissociation at 25°C. of -2229 cal. per mole, a value in gross disagreement with the direct calorimetric determination of -5200 cal. per mole (14). There are indications that the extrapolation methods involved in the E.M.F. procedures break down with moderately strong acids having ionization constants near 10^{-2}.

Optical deviations in solutions of methyl orange can be used also to determine dissociation constants of other moderately strong acids besides bisulfate ion. As with bisulfate ion, changes of optical absorption upon addition of the electrolyte are corrected for neutral salt effects by reference to curves for sodium chloride or barium chloride and the remainder of the deviation is attributed to changes in hydrogen-ion concentration. The results obtained with chloroacetic acid are compared in table 2 with values given in the literature. The agreement between the two optical methods is very good, but both of these differ significantly from the results obtained by classical electrical techniques.

The optical method described in this paper is particularly suitable for the de-

termination of the dissociation constants of the carboxyl groups of the amino acids (27), for the measurements can be made in solutions of very low ionic strength (*ca.* 5×10^{-4}) where the Debye–Hückel limiting law may be used as a close approximation to the activity coefficients of the ions. In consequence the

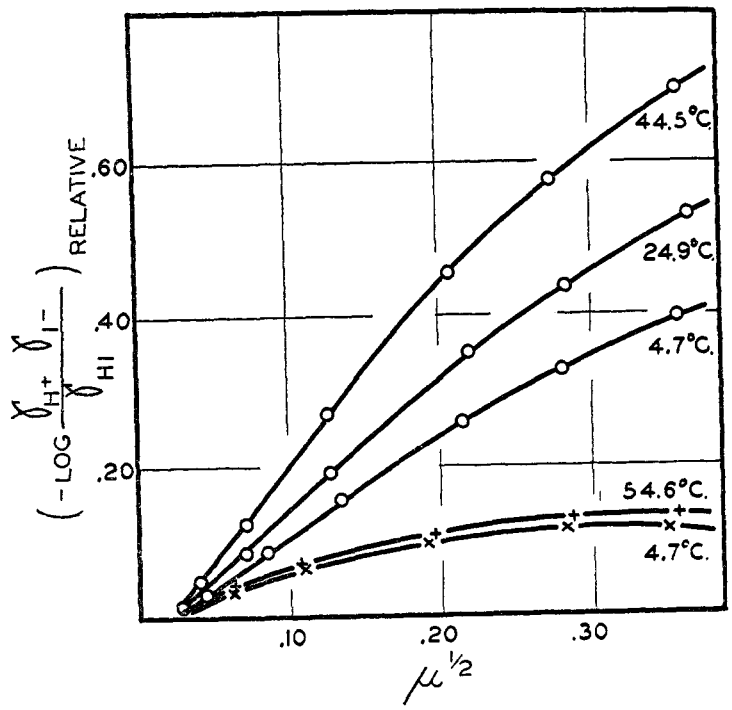


FIG. 5. Salt effect of sodium sulfate on absorption of methyl orange as a function of temperature: ○, sodium sulfate; +, ×, sodium chloride.

TABLE 1

Thermodynamics of ionization of HSO_4^- (*4, 21*)

TEMPERATURE	*K*	ΔF^0	ΔH^0	ΔS^0
°C.		*cal./mole*	*cal./mole*	*cal./mole degree*
5	0.0180	2220	−4270	−23.3
25	0.01015	2720	−5190	−26.5
45	0.00558	3280	−6110	−29.5

extrapolations required involve an almost horizontal line and lead to high precision in the end result (table 3).

III. PROTEIN EFFECTS

Early interest in color changes produced by proteins arose, as in the case of salt effects, in connection with pH determinations (22). Recently it has also

been shown that spectral variations offer a very useful approach to the problem of protein interactions with small molecules (5, 7).

Analysis of the basic causes of protein errors in colorimetric pH determinations indicates that four mechanisms are most important in creating these deviations: (*1*) the protein may disturb micelle equilibria; (*2*) the protein may adsorb an indicator ion of opposite charge, aided by electrostatic attraction, and thereby disturb the equilibrium between the acidic and basic forms of the indicator; (*3*) specific attractive forces may lead to complex formation between the protein and indicator ion even when both have the same electrical charge and hence a strong electrostatic repulsion; and (*4*) the spectrum of the dye in combination with the protein may differ substantially from that of the free dye.

TABLE 2

Ionization constant of chloroacetic acid

METHOD	*K*	TEMPERATURE	REFERENCE
		°C.	
Conductance	1.396×10^{-3}	25.0	(16, 17)
E.M.F.	1.378×10^{-3}	25.0	(26)
Optical (M,K)*	1.337×10^{-3}	25.0	(11)
Optical (Y,K,S)	1.316×10^{-3}	24.8	(4, 21)

* The optical method of Minnick and Kilpatrick depends on a knowledge of the dissociation constant of the indicator. The indicator constant does not enter into our calculations.

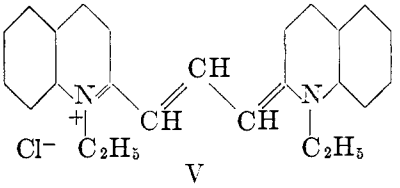
TABLE 3

Ionization constant of carboxyl group of glycine

METHOD	*K*	TEMPERATURE	REFERENCE
		°C.	
E.M.F.	4.47×10^{-3}	25.0	(13)
Optical	4.46×10^{-3}	25.0	(27)

A. Effects on micelle equilibria

A typical example of the disturbance of dye micelle equilibria is illustrated in the work of Sheppard and Geddes (19), exhibited in figure 6. From investigations of deviations from Beer's law, Sheppard and Geddes concluded that the dye pinacyanol (V) exists partially in the dimeric state in aqueous solution.



V

Pinacyanol chloride

The addition of gelatin tends to break up this dimerization and hence changes the spectrum toward that characteristic of the monomer. The addition of the

cationic detergent cetylpyridinium chloride produces an optical effect similar to that of gelatin. Again there is a tendency to break up the dimer. The interaction between dye and protein or detergent, respectively, seems to be between organophile portions of the molecules. Since the cyanine dye is cationic no electrostatic attraction would exist, for the gelatin used was one of high isoelectric point (8.8) and hence it too was cationic at the pH used (5.6).

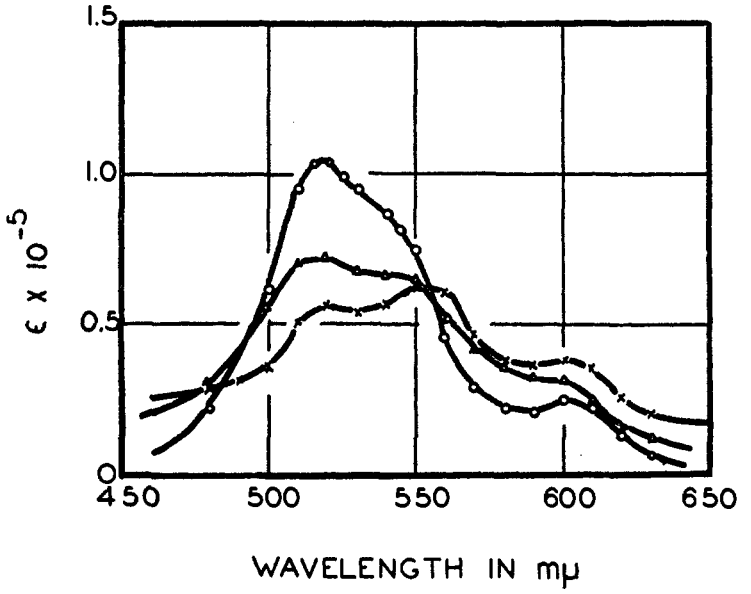


FIG. 6. Absorption spectra of pinacyanol: ○, in aqueous solution; ×, in aqueous solution containing cetylpyridinium chloride; △, in aqueous solution containing gelatin (19).

B. Combination of protein with dye ion of opposite charge

Protein effects on an indicator such as methyl orange cannot be attributed to disturbance of micelle equilibria, since methyl orange obeys Beer's law in dilute solutions (figures 7 and 8). Nevertheless, colorimetric pH determinations with methyl orange in the presence of proteins are frequently grossly in error. The magnitude of such deviations is best illustrated by the work of Thiel and Schulz (24), shown in figure 9. Errors over one pH unit are observed in solutions of egg albumin even at concentrations below 0.5 per cent.

Since methyl orange does not exist in a polymeric state in dilute aqueous solution, the optical deviations in the presence of proteins must be attribtued to a disturbance of the acid–base equilibrium symbolized by equation 1. Such a disturbance seems very plausible, in view of the fact that the isoelectric points of casein and egg albumin are near pH 5. At pH's below 5, both of these proteins would be positively charged and hence would exert a strong electrostatic attraction on the methyl orange anion, I^-. Reference to equation 1 shows that the combination of protein with I^- would decrease the concentration of HI and

hence shift the spectrum toward the anionic form.[3] To return the spectrum toward the protein-free type obviously would require the addition of acid, as has been observed by Thiel and Schulz (24) (figure 9).

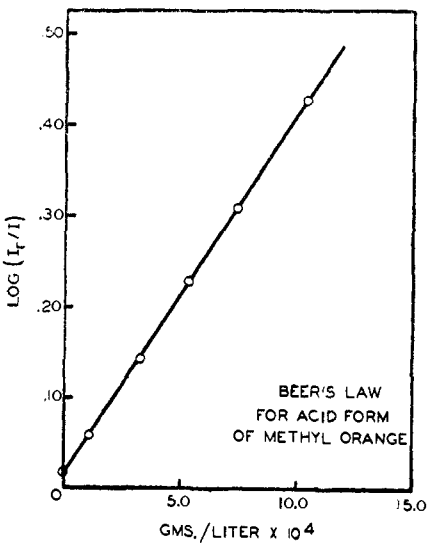


FIG. 7. Optical absorption of methyl orange as a function of concentration, in acid solutions.

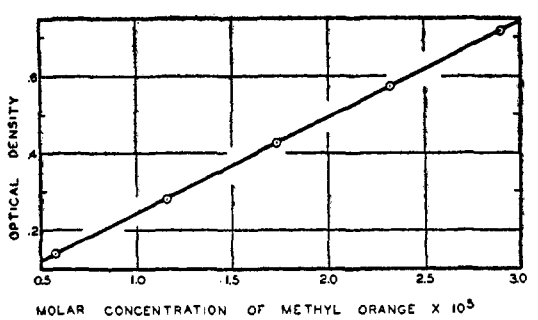


FIG. 8. Optical absorption of methyl orange as a function of concentration in solutions of pH 5.7.

In view of this interpretation of the basis of the spectral changes of methyl orange in the presence of proteins such as egg albumin, one would expect optical deviations to disappear at pH's above 5, since the protein begins to acquire a negative charge. This prediction cannot be tested with methyl orange, because

[3] This assumes that the anion has the same spectrum in the free and in the combined state. As will be shown later, such is not quite the case, but the changes due to combination of methyl orange with the protein are much smaller than those accompanying the addition of a hydrogen ion.

this indicator would be almost completely in the anionic form at pH 5, since its pK is near 3.5. On the other hand, bromocresol green (IV) can be examined in this region, since its pK is 4.92 (12), and in view of the fact that its colored forms

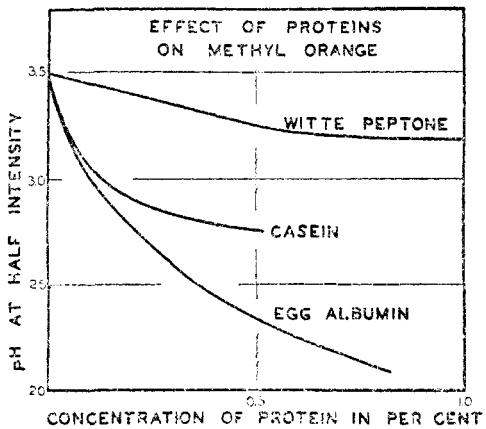


FIG. 9. Colorimetric pH errors of methyl orange in the presence of proteins (24)

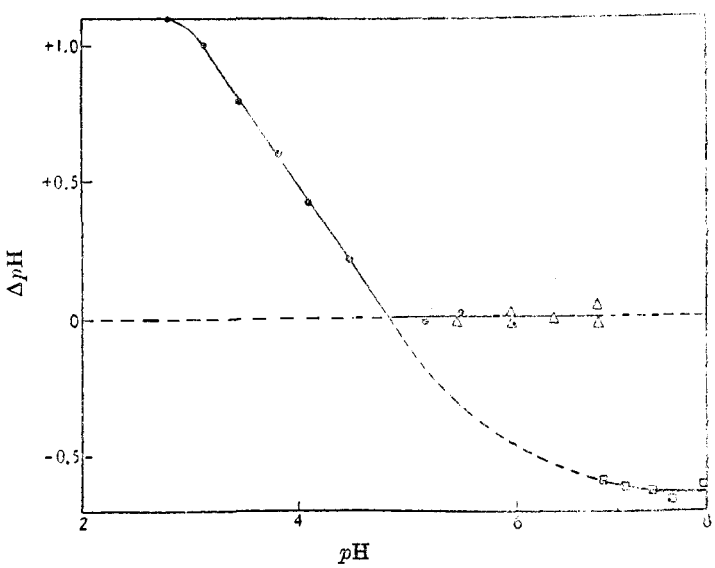
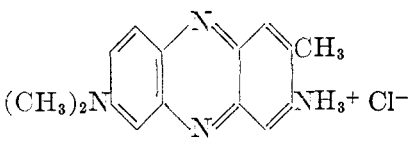


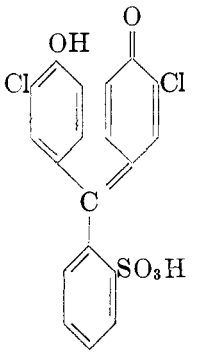
FIG. 10. Colorimetric pH errors in the presence of egg albumin: ●, bromocresol green; △, chlorophenol red; □, neutral red (2).

are anionic, its behavior should be similar to that of methyl orange. The pH error for bromocresol green in the presence of egg albumin has been investigated by Danielli (2). The experimental observations (figure 10) are in agreement with expectation, in that the protein error is large in highly acid solution, decreases with increasing pH, and becomes essentially zero after the isoelectric

point of egg albumin has been attained. Similarly it is interesting to note in figure 10 that neutral red (VI), a cationic indicator, is affected by egg albumin in the pH range above 5 where the protein is negatively charged and can attract



VI
Neutral red

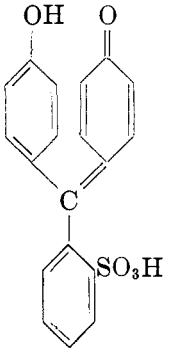


VII
Chlorophenol red

the cation electrostatically. The absence of electrostatic attraction would account for the lack of a protein error in the case of chlorophenol red (VII) (figure 10).

C. Formation of complexes between protein and dye ion of same charge

While the theory of electrostatic attraction of oppositely charged entities accounts for the spectral deviations in the presence of many proteins, there are some situations in which this interpretation is obviously inadequate. Thus, Lepper and Martin (9) have observed an appreciable colorimetric deviation for phenol red (VIII) upon the addition of serum albumin (figure 11). This pro-



VIII
Phenol red

tein is anionic at pH's above 5; hence one would expect no electrostatic interaction with an anionic indicator. Nevertheless the observed colorimetric errors suggest that a specific attraction exists between the serum albumin and this

anionic indicator and that the resultant complex causes a displacement of the acid–base equilibrium.

The possibility of a protein–anion combination even when both species are anionic was investigated by studying the binding of methyl orange anions by serum albumin at pH's above 5. Some typical results obtained by a dialysis–

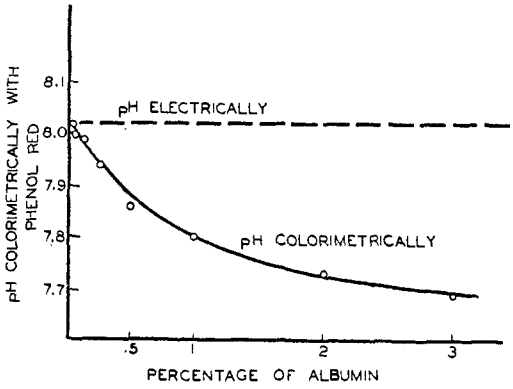


FIG. 11. Colorimetric pH errors of phenol red in the presence of serum albumin (9)

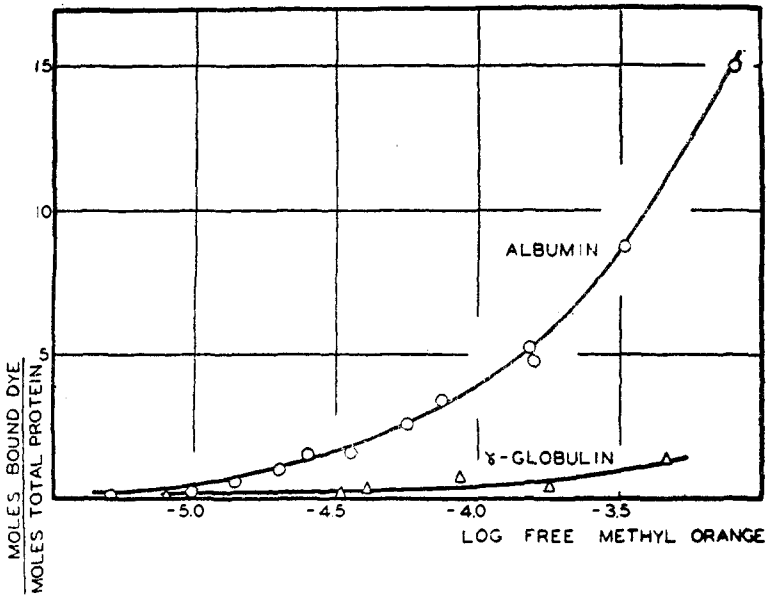


FIG. 12. Binding of methyl orange by serum proteins at pH 5.7

distribution technique are illustrated in figure 12. Clearly, very large fractions of the indicator anion combine to form a complex with the albumin even in solutions of the order of 10^{-5} molar. Such complex formation could have a pronounced effect on the equilibrium between the acid and base forms of an indicator with a pK near the pH range studied.

Formation of complexes between albumin and a dye ion of the same charge is

not limited to methyl orange anions. Quantitative determinations of the degree of binding have been obtained by dialysis–distribution studies with orange I (IX), orange II (X), azosulfathiazole (XI), amaranth (XII), and bromocresol green (IV). Qualitative evidence is available for a large number of other anionic dyes (5, 6).

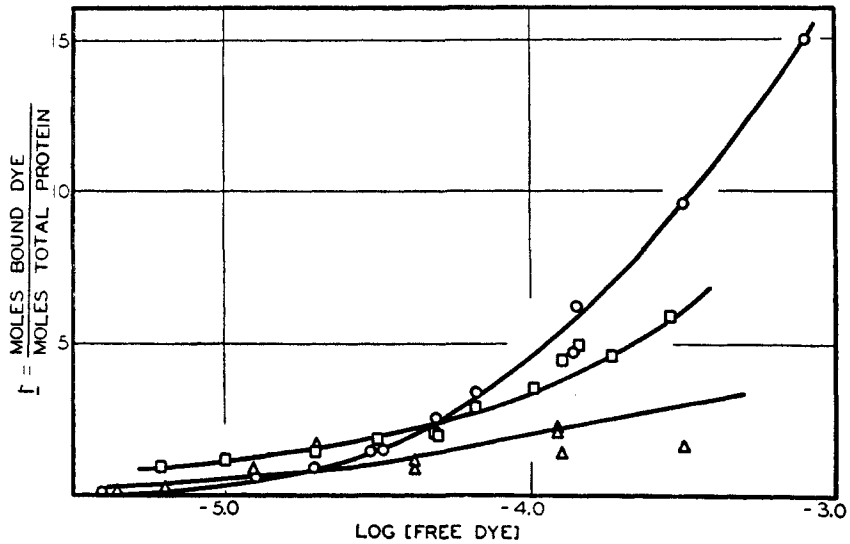
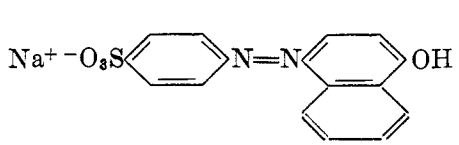
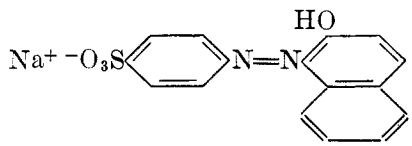


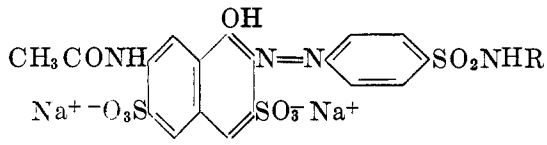
FIG. 13. Binding of sulfonate compounds by bovine serum albumin at pH 5.7; ○, methy orange (II); □, azosulfathiazole (XI); △, amaranth (XII).



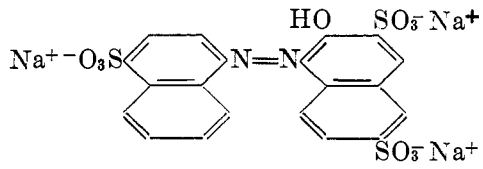
IX
Orange I



X
Orange II



XI
Azosulfathiazole
(R = thiazole ring)



XII
Amaranth

The quantitative data on the binding of methyl orange, azosulfathiazole, and amaranth, respectively, are presented together for comparison in figure 13. One of the most striking characteristics in this figure is the decrease in the curvature as the number of negative charges on the anion increases from 1 to 3. Since decreasing curvature corresponds to less binding relative to that observed in a very dilute solution, it is apparent that the electrostatic repulsion between dye anion and negatively charged albumin becomes increasingly important as the number of charges on the dye increases. Such behavior would be expected, of course, from the principles of electrostatics. In fact, one can express these predictions in quantitative terms if one formulates the proper expression for the law of mass action for such cases of multiple binding.

It is obvious from figures 12 and 13 that more than one molecule of dye may be bound by one protein molecule. If we let P represent the protein and A the dye anion, then the successive equilibria involved may be expressed by the equations

$$\begin{aligned} P + A &= PA \\ PA + A &= PA_2 \\ &\cdots\cdots\cdots \\ PA_{n-1} + A &= PA_n \end{aligned} \tag{5}$$

where n is the maximum number of anions which a protein molecule can bind. The equilibrium constant for any step in equations 5 may be determined from the relation:

$$\frac{(PA_i)}{(PA_{i-1})(A)} = k_i \tag{6}$$

It has been shown (7) that the ratio, r, of the moles of bound anion to the total moles of protein is given, for the general case, by the equation:

$$r = \frac{\sum_{i=1}^{n} i\left(\prod_{j=1}^{i} k_j\right) A^i}{1 + \sum_{i=1}^{n}\left(\prod_{j=1}^{i} k_j\right) A^i} \tag{7}$$

To determine r as a function of the anion concentration, A, it is obviously necessary to have information on the equilibrium constants, k_i.

The simplest situation that one might encounter in protein binding is that in which each anion is bound to the same kind of group on the protein and in which a bound ion exerts no electrostatic influence on succeeding bindings. In such a situation, the strength of attachment would be the same for each bound anion. Hence the relative values of the successive equilibrium constants would be determined solely by statistical factors and would be given by the relation:

$$k_i = \frac{n - (i - 1)}{i} \frac{1}{K} \tag{8}$$

K is a specific binding constant which depends on the nature of the anion as well as on the character of the protein. Where the statistical effect is predomi-

nant and the equilibrium constants are given by equation 8, the relatively involved equation 7 may be reduced to a very simple linear form:

$$\frac{1}{r} = \frac{1}{n}\left[\frac{K}{(\mathrm{A})} - 1\right] \tag{9}$$

This statistical relation is a very good approximation to the quantitative data on the binding of the singly charged anionic dyes, methyl orange, orange I, and orange II, respectively, as is evident from figure 14. Apparently each focus of attachment on the albumin molecule must have the same inherent tendency for attaching an anion of any given one of these dyes.

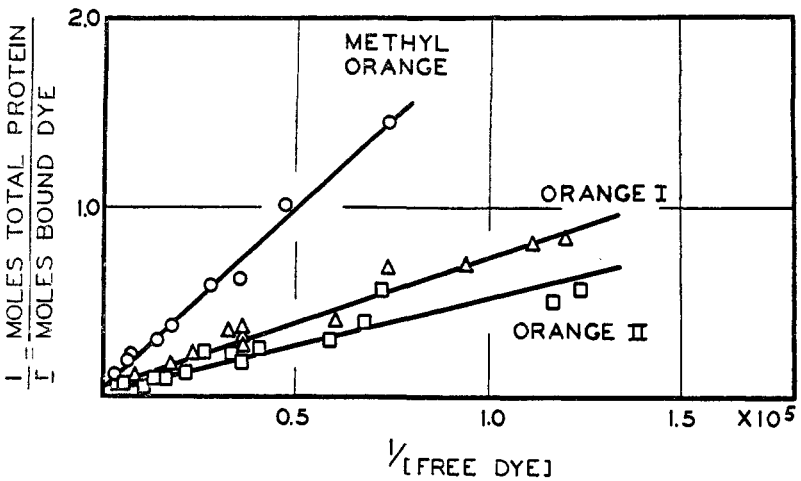


FIG. 14. Binding of some singly charged dyes by serum albumin at pH 5.7

For dyes with two and three charged groups, the statistical distribution of constants is unable to account for the actual behavior. It is necessary to take electrostatic interactions into account. It is perhaps easiest to visualize the nature of the electrostatic effect by introducing it in terms of the free energy of the binding process for the i^{th} step:

$$\Delta F^0 = -RT \ln k_i = \Delta F_{\text{stat}} + \Delta F_{\text{specific}} + \Delta F_{\text{elect}} \tag{10}$$

ΔF_{stat} represents the statistical contribution to the change in free energy; ΔF_{spec} the contribution of the intrinsic binding constant K; and ΔF_{elect}, the contribution of electrostatic interactions. The electrostatic contribution may be evaluated in terms of the charging energy of an ion in an electrolyte solution. To focus attention on the effect of the charge of the anion alone it is convenient to consider the transfer of one dye ion from one complex to another of lower degree, as is represented in the equation:

$$\mathrm{PA}_i + \mathrm{PA}_{i-2} = 2\mathrm{PA}_{i-1} \tag{11}$$

For this reaction the electrostatic free-energy change is given by the expression

$$\Delta F_{\text{elect}} = -\frac{Nz^2e^2}{D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) \tag{12}$$

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

$$\kappa = \left(\frac{4\pi Ne^2}{1000Dk'T}\right)^{\frac{1}{2}} \Gamma^{\frac{1}{2}} \tag{13}$$

where k' is the Boltzmann constant, T is the absolute temperature, and Γ is twice the ionic strength of the medium.

From equation 12 it is clear that the electrostatic effect, depending on the square of z, becomes increasingly important with increasing charge on the anion. Since the anion and protein have the same charge, the electrostatic interaction is a repulsive one and the protein tends to bind fewer of the more highly charged anions, as the anion concentration increases. Thus the curvature of the graphs in figure 13 should be less for azosulfathiazole (two SO_3^-'s) than for methyl orange (one SO_3^-), and that for amaranth (three SO_3^-'s) should be smallest. The observed behavior is not only in qualitative agreement with the theoretical explanation but also quite close quantitatively. By making reasonable choices for the radii of the protein and of the dye anions it is possible to calculate the electrostatic free-energy change from equation 12. This information, in addition to an empirical evaluation of a single constant, K, the intrinsic binding constant, enables one to evaluate the individual constants, k_i, and ultimately r, the moles of bound anion per mole of total protein. Quite satisfactory agreement has been found between the theoretical curves so calculated and the observed experimental values.

D. Changes in spectra of dye–protein complexes

1. Dependence on nature of dye

In addition to displacing acid–base equilibria in indicators, proteins may cause optical deviations by changing the spectrum of the bound form of the dye. Such an alteration is illustrated in figure 15 for methyl orange in the presence of serum albumin. While the displacement is not great, there is a distinct lowering of the maximum absorption and a shift of the peak by about 200 A. Similar results are obtained with orange I (figure 16), azosulfathiazole (figure 17), and amaranth (figure 18). While the addition of bovine albumin lowers the value of the maximum extinction coefficient in each of the above azo compounds, the shift in the wave length of the absorption peak is not the same in each case (table 4). Thus the complexes with azosulfathiazole and amaranth, respectively, have their peaks shifted to longer wave lengths, whereas those with orange I and orange II, respectively, show 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.

It is important, of course, to prove that these spectral displacements are due to actual binding of the dye anion by the serum albumin. Since an alternative explanation would be the displacement of the polymer equilibria by the protein,

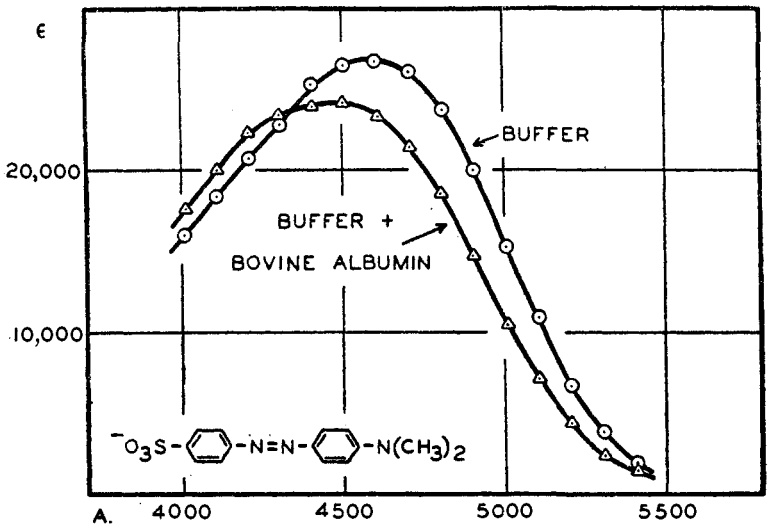


FIG. 15. Effect of serum albumin on spectrum of base form of methyl orange

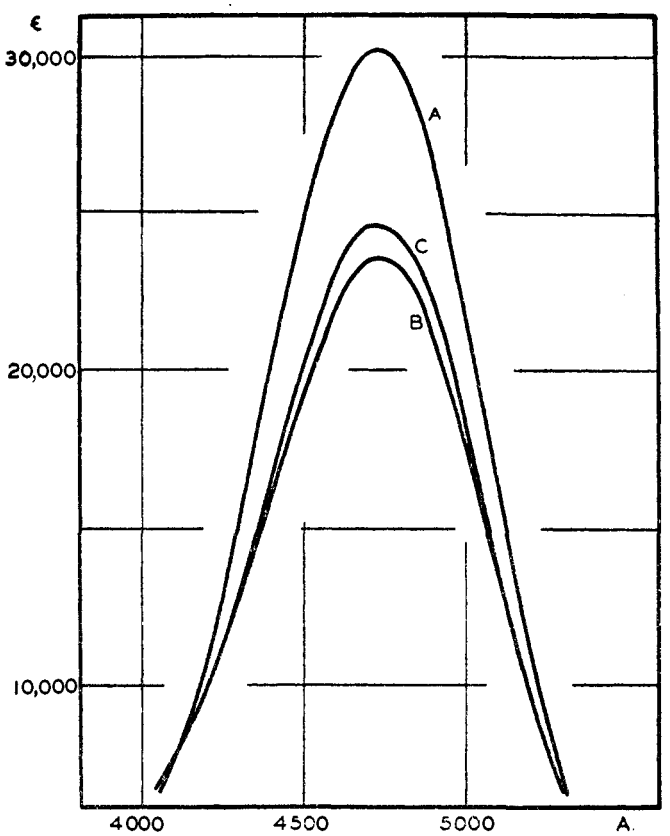


FIG. 16. Absorption spectra of Orange I: A, in buffer at pH 6.8; B, in buffer containing bovine albumin; C, in buffer containing bovine albumin and potassium acid phthalate.

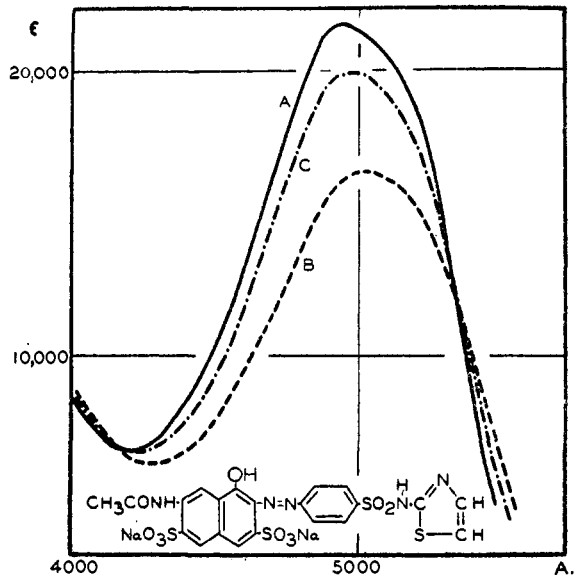


FIG. 17. Absorption spectra of azosulfathiazole: A, in buffer at pH 6.8; B, in buffer containing bovine albumin; C, in buffer containing bovine albumin and potassium acid phthalate.

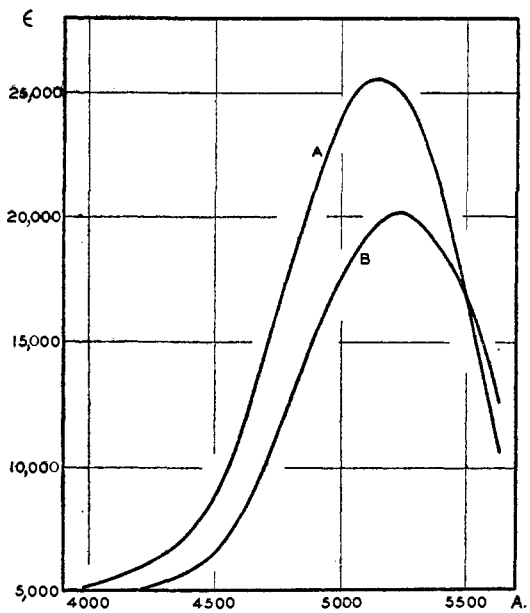


FIG. 18. Absorption spectra of amaranth: A, in buffer at pH 6.9; B, in buffer containing bovine albumin.

the adherence to Beer's law was investigated for each dye. Linear dependence of optical absorption upon concentration was observed in every case up to concentrations of at least 10^{-4} molar; hence the possibility of dimerization has been

ruled out. This interpretation also seemed unlikely when it was found that micellar sodium dodecyl sulfate did not produce spectral displacements even in 1 per cent concentration, a quantity which is almost five times as much as is necessary to produce a maximum effect with bovine albumin.

TABLE 4

Effect of bovine serum albumin on the position of absorption peak

DYE	ABSORPTION PEAK IN BUFFER	ABSORPTION PEAK IN PRESENCE OF PROTEIN
	A.	*A.*
Methyl orange	4600	4400
Orange I	4730	4730
Orange II	4810	4810
Azosulfathiazole	4950	5050
Amaranth	5140	5240

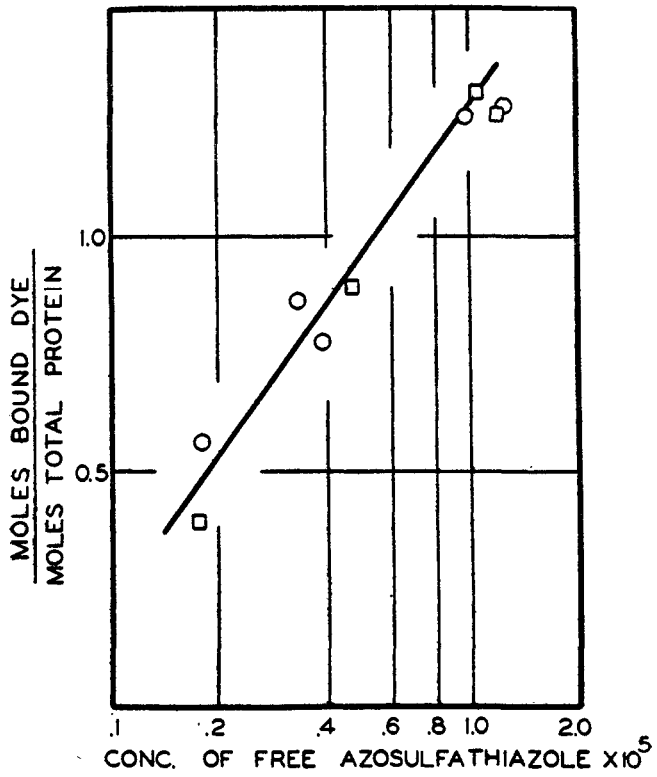
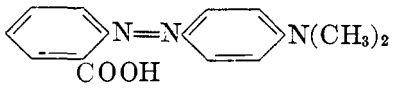


FIG. 19. Quantitative data on the extent of binding of azosulfathiazole: ○, from spectral measurements; □, from dialysis–equilibrium studies.

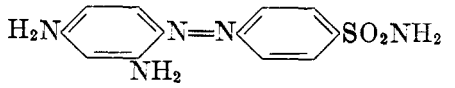
Direct evidence that the spectral displacements are due to binding was also obtained by calculating the extent of binding from optical changes in solutions of varying protein and dye concentrations (5). The results obtained in this fashion agree very well (figure 19) with those obtained by the direct dialysis–

distribution method. Thus there is no question but that the spectral shifts are a result of formation of complexes between the dye and protein molecules.

It is not essential for binding by albumin that the dye have a sulfonate group. Similar results (figure 20) are obtained with methyl red (XIII), which has a carboxyl group. Apparently, however, an anionic substituent is essential for *strong* binding by albumin, for prontosil (XIV), which lacks such a group, shows only small spectral changes.



XIII
Methyl red



XIV
Prontosil

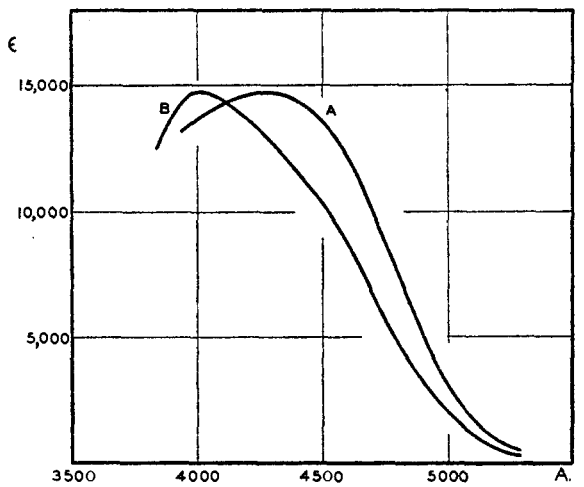


FIG. 20. Absorption spectra of methyl red: A, in buffer at pH 7.3; B, in buffer containing bovine albumin.

So far, spectral deviations have been observed only with serum albumin, bovine or human. No significant effects are obtained with γ-globulin, gelatin, egg albumin, or casein. Apparently these proteins, basic to their isoelectric points, are incapable of binding the dyes. The absence of appreciable binding has been confirmed for γ-globulin by dialysis techniques. Only in concentrated solutions is a significant quantity of methyl orange bound (figure 12) and even then the fraction in the complex is only a small part of that free in solution.

2. Dependence on other substances in solution

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. This reversal has been observed with each of the dyes investigated and is illustrated for orange I and azosulfathiazole in figures 16 and 17. Apparently the organic

acid displaces the dye from its protein complex. The carboxyl ion and the sulfonate ion thus must compete for the points of attachment on the protein molecule.

A survey of the competitive abilities of a number of organic acids against azosulfathiazole is summarized in table 5. 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. From the data listed in this table it is quite clear that optical deviations depend not only on the nature of the protein but also on the nature of the buffer.

TABLE 5

Competition between acids and azosulfathiazole (5)

COMPOUND	CONCENTRATION	pH	$\Delta\epsilon$ AT MAXIMA	PER CENT COMPOUND IN FORM OF ANION
	moles/liter			
p-Chlorobenzoic acid	1.18×10^{-2}	6.51	5100	100
o-Chlorobenzoic acid	1.18	6.51	4500	100
o-Aminobenzoic acid	1.35	6.45	4500	97
p-Aminobenzoic acid	1.34	6.45	2000	97
	1.44	9.02	0	100
Sulfanilic acid	0.96	6.55	1400	100
Sulfanilamide	1.31	5.12	0	0
Sulfamic acid	2.06	8.81	0	100
Salicylic acid	1.47	8.98	3100	100
Potassium acid phthalate	1.98	4.79	4300	75 A^-, 24 A^{--}
	0.49	5.42	3700	41 A^-, 59 A^{--}
	0.38	9.02	400	100 A^{--}
Maleic acid	1.60	5.82	3000	64 A^-, 36 A^{--}
	1.65	8.64	200	100 A^{--}
Succinic acid	1.71	4.38	0	58 A^-, 5 A^{--}
	1.65	8.72	0	100 A^{--}
Veronal	1.12	5.12	0	0

Thus phthalate buffers would reduce the protein errors very appreciably, particularly at low pH's. Similarly, if an organic metabolite is present in a medium of biological importance, the optical shifts due to the presence of protein may be quite different from those observed in the absence of the organic compound.

3. Dependence on pH

In the case of azosulfathiazole, the difference in heights of the absorption peaks in the absence and presence of albumin did not vary significantly over a pH range of 5 to 9. No studies could be made at higher pH's, since the interpretation of spectral changes in this dye is complicated by the ionization of the phenolic hydroxyl group.

With methyl orange, however, investigations can be made beyond pH 9 and lead to the striking results illustrated in figure 21. Clearly there is a decrease in the optical deviation due to the presence of protein as the pH is increased,

with complete disappearance of any effect at pH's slightly above 11. Such behavior suggests that binding by the protein disappears at high pH's. This prediction has been confirmed by ultrafiltration[4] studies. Methyl orange is not bound by bovine albumin to any significant extent at pH's approaching 12.

It is also of interest to note that the reversing ability of a given carboxylic acid decreases with increase in pH. The trend is quite clear from the data in table 5 on potassium acid phthalate, *p*-aminobenzoic acid, and maleic acid, respectively. The decrease in affinity at high pH must be an expression of the increasing electrostatic repulsion of the organic ion, due to the increasing negative charge on the protein molecule.

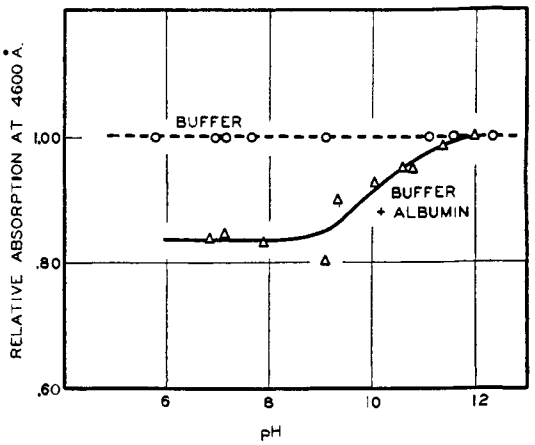


FIG. 21. Effect of pH on absorption spectra of methyl orange in albumin solutions

E. Nature of the protein–anion bond

The strength of the protein–anion bond, or the value of the intrinsic binding constant, K, seems to be determined by two factors, an electrostatic attraction and van der Waals interactions. The basis of an electrostatic attraction becomes evident if one considers the amino acid composition of a protein such as serum albumin. A very abbreviated schematic diagram of the structure of a protein is indicated in figure 22. At a pH of about 6, the basic amino acids, histidine, lysine, and arginine, are combined with a hydrogen ion and hence have a net positive charge, whereas the acidic amino acids, such as glutamic acid, have released a hydrogen ion and formed carboxylate ions. Since the isoelectric point of serum albumin is near pH 5, the number of carboxylate ions at pH 6 exceeds the number of quaternary nitrogen atoms and hence the net charge on the protein molecule is negative. Nevertheless, the presence of positively charged nitrogen atoms forms a focus of attachment for the negative sulfonate or carboxylate substituents of the dyes and small organic anions. A similar picture has been drawn to interpret the interactions of detergents with proteins (10, 15).

[4] The dialysis–distribution method was avoided because the time required for equilibrium (72 hr.) may be sufficient to cause extensive denaturation of the protein at high pH's.

In the case of the dye–protein combinations described here, the electrostatic interactions seem to involve largely the lysine group of the protein. For on the basis of the picture outlined one would expect the binding to disappear at pH's where the quaternary nitrogen atoms lose their hydrogen ions and hence their positive charge. These pH's are listed in table 6. The region of transition in figure 21 corresponds rather well to the region in which the ε-ammonium group of lysine loses its hydrogen ion.

It is also evident from the experiments described that electrostatic interactions alone will not account for the relative behavior of different dyes and organic anions in combining with proteins. Thus, for example, the intrinsic affinity con-

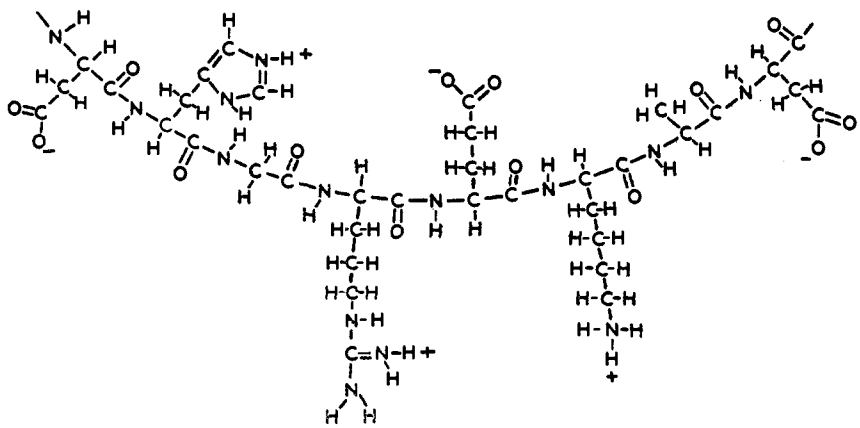


FIG. 22. Abbreviated schematic diagram of a protein molecule

TABLE 6

Acidity constants of basic groups in proteins (1)

AMINO ACID	BASIC GROUP	pK (25°C.)
Histidine	Imidazolium	5.6–7.0
Lysine	ε-Ammonium	9.4–10.6
Arginine	Guanidinium	11.6–12.6

stant for azosulfathiazole (XI) and bovine albumin is greater than that for methyl orange (II) by an amount corresponding to over 500 cal. in ΔF^0. Since only one sulfonate group seems to be involved in the binding in each case, the additional intrinsic affinity which the protein has for azosulfathiazole must be attributed to the additional groups in this molecule. Since there are many added substituents it is impossible to single out one as most important, although it does seem likely that the van der Waals interaction of the added aromatic ring contributes the major portion of the extra stabilization energy.

Similarly, in the competition of small molecules with large dyes for binding positions on the protein it is evident that increasing pH puts the small molecules at a greater disadvantage. Apparently van der Waals forces contribute more

to the stability of the protein–dye complexes than to that of the organic anion complexes, since the latter have at most but one aromatic ring. Hence at high pH's electrostatic repulsion is more effective against the organic anions than against the dyes, and therefore the competing ability of the former decreases.

Competition phenomena also show that van der Waals interactions alone are insufficient to maintain a stable bond in the complex. This seems evident from the fact that the undissociated organic acid is much less effective as a displacing agent than is the anion.

IV. CONCLUSIONS

The results of the investigations described here indicate that the effects of salts or proteins on indicator and dye spectra can be accounted for largely in terms of electrostatic interactions. These interactions may be of the Debye–Hückel ion–cloud type or may involve actual complex formation, as in the case of proteins. Where such interactions can be separated from other effects, the experimental data may be accounted for quantitatively by thermodynamic methods with the aid of the fundamental theory of electrostatics. For both salts and proteins, however, certain specific interactions are also encountered. For electrolytes, these effects may be visualized as ion–dipole interactions with the solvent molecules. In the case of the proteins, specificity phenomena must be related to the internal structure of these large molecules. The emphasis in these spectral studies is shifting, therefore, from the dye to the electrolyte and to the proteins, for these investigations offer another method of probing into the nature of these substances in aqueous solution.

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