Acknowledgments.—The authors are indebted to Dr. Gladys Hobby for the acute toxicity determinations and to Dr. Ben Sobin for the bioassays. The analytical values were determined by Dr. John Means, Mrs. Edwina Wolke and Mrs. Viola Stafford. The authors are grateful for the technical assistance of Mr. Michael Marmo.

Summary

Streptomycylamine, N′-*n*-octyl, *n*-decyl, *n*-dodecyl, *n*-tetradecyl and *n*-hexadecylstreptomycylamine have been prepared, characterized and shown to have antibacterial activity.

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[CONTRIBUTION FROM THE BIOCHEMICAL LABORATORY, DEPARTMENT OF CHEMISTRY, STANFORD UNIVERSITY]

The Combination of Organic Anions with Serum Albumin. VII. The Protein Sites Involved in the Combination

BY J. D. TERESI

The study of complex formation of serum albumin with organic anions of known structure offers promise of obtaining information of protein configuration. Serum albumins are especially suited for this type of study since these proteins have been shown to combine reversibly with many types of organic anions.[1-7]

A large amount of evidence has been reported to support the view that anions combine with positive groups on the albumin molecule. Therefore, anion–protein interaction at *p*H 7.6 would involve, for the most part, the guanidinium groups of the arginine residues and the ε-ammonium groups of the lysine residues of the albumin molecule. The study of these interactions after modification of the protein molecule by removal of either of these positive charges may give information concerning the nature and the identification of the binding sites. In this paper the results of anion binding with native and modified bovine serum albumin are reported.

Experimental

Native Albumin.—The crystalline bovine serum albumin used was obtained from Armour Laboratories. The water content was obtained by a dry weight determination on a sample of protein dried at 110° for twenty-four hours.

Modified Albumin.—One modified protein was prepared by acetylation of the ε-ammonium groups of the lysine residues by treating one part of the bovine serum albumin in one-half saturated sodium acetate with 1.2 parts of acetic anhydride at 0 to 5° as described by Olcott and Fraenkel-Conrat.[8] The solution was then dialyzed exhaustively and the protein was then lyophilized. The resulting product was soluble in water. A formal titration[9] indicated that 90 to 95% of the ε-amino groups were acetylated.

Another modified protein was prepared by treating the bovine serum albumin with excess 2 *M* formaldehyde for ten minutes at room temperature and at *p*H 11 with subsequent exhaustive dialysis to remove free and reversible formaldehyde. Under these conditions formaldehyde reacts reversibly with ε-ammonium groups and irreversibly with guanidinium groups.[8] This was verified by the formal titration method which showed that 95–100% of the ε-amino groups were still available. This water-soluble protein was also lyophilized and stored for later use.

Solutions.—Protein solutions of desired concentration and ionic strength (0.2) were prepared with phosphate buffer at *p*H 7.6. Solutions of the sodium salts of the compounds reported in this paper were also made up to the desired concentrations with phosphate buffer at *p*H 7.6 and ionic strength 0.2.

Spectral Method.—The spectrophotometric behavior of *o*-nitrophenolate, *p*-nitrophenolate, *m*-nitrophenolate, picrate and methyl orange was observed at *p*H 7.6 and ionic strength 0.2 in the presence of buffer, native albumin and modified albumin with the aid of the Beckman spectrophotometer. The concentration of the protein solution used in each case was approximately 0.35%.

Dialysis.—Commercial sausage casing was used in the preparation of cellophane bags for the dialysis-equilibrium studies. Cellophane bags containing 5 ml. of 0.2 or 0.4% buffered protein solution were immersed in 10 ml. of each anion solution contained in a suitable bottle. Controls containing buffer only inside the bag were prepared in the same manner for each concentration of anion. The bottles were placed in a cold room at 1° for three days. The bags were then removed and the external solutions were analyzed spectrophotometrically for the anion.

Results and Discussion

Spectral Studies.—The spectroscopic behavior of a buffered methyl orange solution is shown by Curve 4 of Fig. 1, which was obtained by plotting the wave length in ångström units against the optical density expressed as log I_0/I. The addition of buffered native serum albumin to the methyl orange solution alters the spectrum of methyl orange as can be seen by comparing Curve 4 with Curve 1 of Fig. 1. This displacement has been interpreted by Klotz[10] as an effect due to the formation of a methyl orange–albumin complex. The substitution of acetylated albumin (Curve 2, Fig. 1) or formaldehyde treated albumin (Curve 3, Fig. 1) for the native albumin results in a reversal of the displacement. The experiment represented by Curve 3 was repeated with a carefully treated al-

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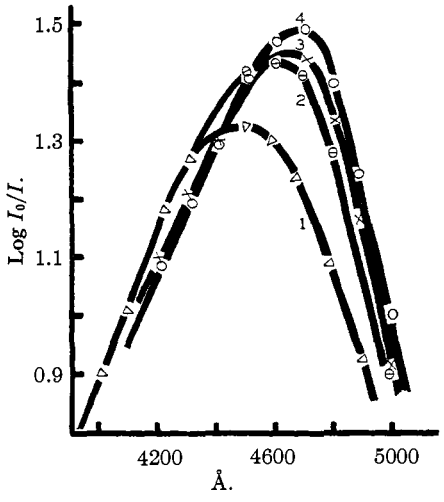

Fig. 1.—Spectrophotometric data for methyl orange: 1, in the presence of native albumin; 2, in the presence of acetylated albumin; 3, in the presence of formaldehyde treated albumin; 4, in the absence of protein.

bumin in which all the ϵ-amino groups were still free to react, and the wave length of maximum absorption was found to be 4500 instead of 4680 Å. However, the value of the optical density still indicated a reversal of the displacement as described above. Since acetylation removes the ϵ-amino positive charge on the protein and formaldehyde treatment removes the guanidyl positive charge, the results from spectral data seem to indicate that complex formation involves both the ϵ-ammonium groups of lysine and the guanidinium groups of the arginine residues. The extent of the involvement of each group will be discussed in connection with the dialysis-equilibrium experiments.

The spectral displacement curves of *p* and *m*-nitrophenolates and the reversal curves produced by the modified albumins are shown in Figs. 2 and 3. The curves obtained in the presence of acetylated albumin are identical to those in the presence of buffer. Again treatment of the guanidyl groups as described resulted in an appreciable change in the spectral displacement. The reason for the decrease in optical density shown in Curve 4 of Fig. 2 is not clear at this time. The spectra of *m*-nitrophenolate in the presence of native serum albumin (Curve 1, Fig. 3) shows a large increase in optical density which is not shown in the cases of the modified proteins. This same tendency is exhibited to a lesser degree in the case of *p*- and *o*-nitrophenolates and picrate. The reason for this phenomenon is obscure at the present time. It is known that an increase in ionization results in an increase in the optical density of *m*-, *o*- and *p*-nitrophenolates. Since picrate is totally ionized, it is difficult to attempt an explanation of the observed optical density increases on the basis of ionization changes at a constant *p*H. Although the effects on the spectral behavior by modified albumins are qualitative, it is evident

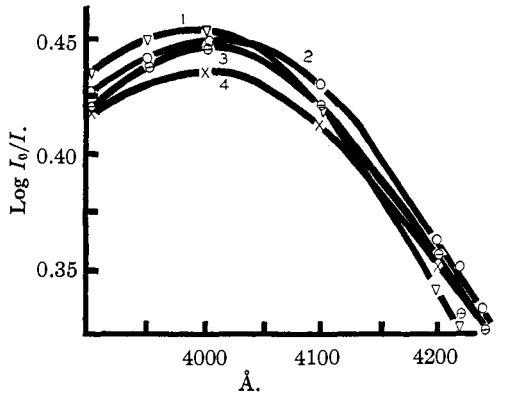

Fig. 2.—Spectrophotometric data for *p*-nitrophenolate: 1, in the presence of native albumin; 2, in the absence of protein; 3, in the presence of acetylated albumin; 4, in the presence of formaldehyde treated albumin.

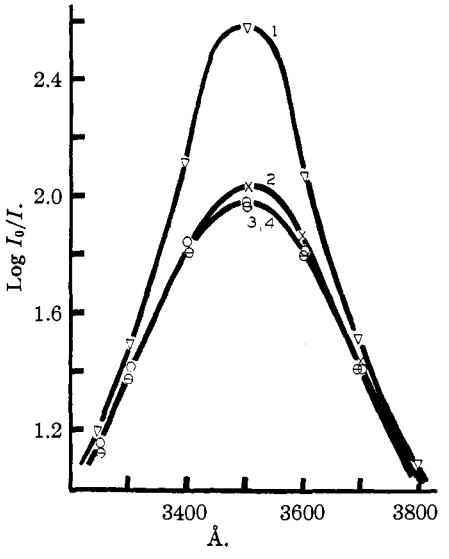

Fig. 3.—Spectrophotometric data for *m*-nitrophenolate: 1, in the presence of native albumin; 2, in the presence of formaldehyde treated albumin; 3, in the absence of protein; 4, in the presence of acetylated albumin.

that ϵ-ammonium groups are necessary for the complete binding of methyl orange, *p*-nitrophenolate and *m*-nitrophenolate. The importance of the guanidyl groups is also indicated, but the extent of involvement of these groups is not clear at this point and will be considered in the discussion of the results of the dialysis-equilibrium experiments.

In the cases of the ortho substituted nitrophenolate anions as represented by *o*-nitrophenolate and picrate (2,4,6-trinitrophenolate), the guanidyl groups of the arginine residues are important sites of combination as the spectral curves shown in Figs. 4 and 5 indicate. The decrease in optical density caused by acetylated albumin without displacement of the wave length maximum in the picrate spectrum as shown in

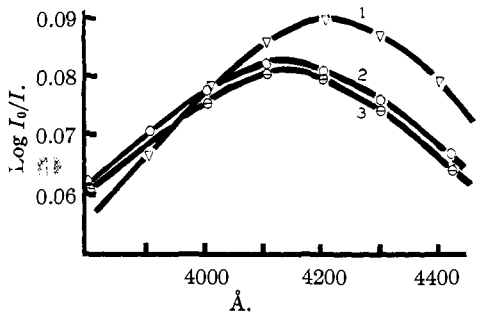

Fig. 4.—Spectrophotometric data for *o*-nitrophenolate: 1, in the presence of native albumin; 2, in the absence of protein; 3, in the presence of formaldehyde treated albumin.

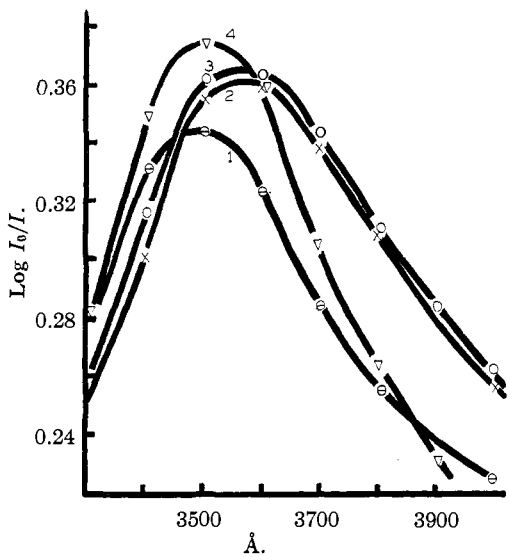

Fig. 5.—Spectrophotometric data for picrate: 1, in the presence of acetylated albumin; 2, in the presence of formaldehyde treated albumin; 3, in the absence of protein; 4, in the presence of native albumin.

Fig. 5 (Curve 1) cannot be explained at this time. Whether or not this constancy of wave length maximum indicates the lack of interaction between anion and the ε-ammonium group is not evident from the spectral data. The involvement of these groups in the picrate binding will be clarified somewhat by the data obtained with the quantitative equilibrium-dialysis experiments. The spectral data of the anions studied are given in Table I showing λ of maximum absorption, anion concentration, optical density and amount of ionization of each compound.

Equilibrium-Dialysis.—The values $1/r$ and $1/c$ used in the graphical representation of the data were calculated in the manner described in a previous paper.[6] The symbol r represents the ratio of moles of bound anion to moles of protein and c represents the free anion concentration. The results of typical dialysis-equilibrium experiments are shown in Tables II–VI. In Fig. 6 is reported the summary of the binding data for methyl orange and bovine serum albumin under

TABLE I

SPECTRAL DATA OF ANIONS IN PRESENCE OF BUFFER, NATIVE ALBUMIN AND MODIFIED ALBUMIN, Γ/2 = 0.2, *p*H 7.6

	Concn. × 10^5	$\lambda_{max.}$, Å.	Optical density log I_0/I	Ionization of compound, %
o-Nitrophenolate + buffer	2.77	4100	0.083	75
o-Nitrophenolate + F Pr[a]	2.77	4100	.081	
o-Nitrophenolate + N Pr	2.77	4200	.09	
Picrate + buffer	2.5	3575	.365	100
Picrate + N Pr	2.5	3500	.375	
Picrate + Ac Pr	2.5	3470	.345	
Picrate + F Pr	2.5	3575	.363	
p-Nitrophenolate + buffer	3.4	4030	.453	72.5
p-Nitrophenolate + F Pr	3.4	4030	.430	
p-Nitrophenolate + N Pr	3.4	3990	.455	
p-Nitrophenolate + Ac Pr	3.4	4010	.450	
m-Nitrophenolate + buffer	166.7	3500	1.98	16
m-Nitrophenolate + F Pr	166.7	3500	1.98	
m-Nitrophenolate + Ac Pr	166.7	3500	2.02	
m-Nitrophenolate + N Pr	166.7	3500	2.60	
Methyl orange + buffer	6.0	4700	1.51	100
Methyl orange + Ac Pr	6.0	4600	1.44	
Methyl orange + F Pr	6.0	4680	1.40	
Methyl orange + N Pr	6.0	4500	1.33	

[a] F Pr, N Pr, Ac Pr denote formaldehyde treated protein, native protein and acetylated protein, respectively.

TABLE II

BINDING OF 2,4-DICHLOROPHENOLATE ANION BY NATIVE BOVINE SERUM ALBUMIN AT 1° IN PHOSPHATE BUFFER, Γ/2 = 0.2, *p*H 7.6

Equil. concn. without prot., m./l. × 10^5	Equil. concn. with prot., m./l. × 10^5	Moles bound anion × 10^7	Moles bound anion per mole prot.,[a] r	$1/r$	$1/c$ × 10^{-4}
164.0	150.0	21.0	14.0	0.072	0.067
161.7	149.4	18.5	12.9	.078	.067
132.3	119.4	19.35	12.9	.078	.084
128.4	116.4	18.0	12.6	.08	.086
99.9	88.5	17.1	11.4	.088	.113
93.3	81.6	17.6	12.3	.0814	.124
67.8	57.6	15.3	10.2	.098	.173
64.0	53.0	15.8	11.0	.091	.187
34.3	28.0	9.45	6.3	.16	.354
32.8	26.3	9.8	6.5	.154	.38
31.7	25.6	9.1	6.1	.164	.39
21.1	17.0	6.2	4.1	.244	.59
17.3	13.7	5.4	3.6	.278	.73
16.0	11.9	6.2	4.1	.244	.84
14.3	11.0	5.0	3.3	.303	.91
13.1	9.3	5.7	3.8	.26	1.07
11.0	8.05	4.43	3.0	.34	1.3
9.5	6.3	4.8	3.2	.313	1.6
6.3	4.05	3.33	2.2	.45	2.4

[a] The molecular weight of serum albumin was assumed to be 70,000; 5 ml. of protein solution containing 1.5 × 10^{-7} mole.

TABLE III

BINDING OF 2,4-DICHLOROPHENOLATE BY ACETYLATED BOVINE SERUM ALBUMIN AT 1° IN PHOSPHATE BUFFER, $\Gamma/2 = 0.2$, pH 7.6

Equil. concn. without prot., m./l. × 10^5	Equil. concn. with prot., m./l. × 10^5	Moles bound anion × 10^7	Moles bound anion per mole prot., r	$1/r$	$1/c$ × 10^{-4}	Mole prot., × 10^7
266	226	60.0	21.0	0.048	0.044	2.86
198.0	161.0	55.5	19.8	.055	.063	2.86
128.0	100.0	42.0	14.7	.068	.10	2.86
67.5	49.0	27.8	9.7	.103	.20	2.86
64.55	55.32	13.82	9.67	.104	.181	1.43
52.2	38.0	21.3	7.45	.134	.263	2.86
38.0	26.0	18.0	6.3	.159	.385	2.86
37.61	31.51	9.15	6.4	.156	.318	1.43
27.5	18.0	14.2	5.0	.200	.56	2.86
25.98	21.27	7.06	4.95	.202	.47	1.43
22.9	18.33	6.86	4.8	.209	.545	1.43
19.65	15.8	5.77	4.04	.248	.633	1.43
16.78	13.5	4.92	3.44	.291	.74	1.43
14.2	7.0	10.8	3.78	.264	1.43	2.86
13.25	10.19	4.56	3.19	.314	0.982	1.43
13.2	8.0	7.8	3.07	.326	1.25	2.86

TABLE IV

BINDING OF METHYL ORANGE BY NATIVE BOVINE SERUM ALBUMIN AT 1° IN PHOSPHATE BUFFER, $\Gamma/2 = 0.2$, pH 7.6

Equil. concn. without prot., m./l. × 10^5	Equil. concn. with prot., m./l. × 10^5	Moles bound anion × 10^7	Moles bound anion per mole prot.,[a] r	$1/r$	$1/c$ × 10^{-4}
30.48	18.06	18.60	6.50	0.154	0.56
8.82	4.02	7.20	2.52	.40	2.50
6.12	2.88	4.80	1.70	.59	3.50
4.47	2.00	3.70	1.30	.77	5.00
3.02	1.37	2.48	0.87	1.15	7.30
2.35	1.08	1.91	0.67	1.50	9.25
1.47	0.67	1.20	0.41	2.44	14.90

[a] 5 ml. of protein solution contains 2.86×10^{-7} mole.

TABLE V

BINDING OF METHYL ORANGE BY FORMALDEHYDE TREATED BOVINE SERUM ALBUMIN AT 1° IN PHOSPHATE BUFFER, $\Gamma/2 = 0.2$, pH 7.6

Equil. concn. without prot., m./l. × 10^5	Equil. concn. with prot., m./l. × 10^5	Moles bound anion × 10^7	Moles bound anion per mole prot.,[a] r	$1/r$	$1/c$ × 10^{-4}
14.63	8.75	8.80	3.08	0.324	1.14
11.10	6.60	6.70	2.39	.420	1.52
5.97	3.40	3.86	1.35	.740	2.95
4.51	2.47	3.06	1.07	.935	4.10
3.00	1.61	2.08	0.73	1.370	6.20
1.57	0.79	1.17	0.41	2.44	12.60

[a] 5 ml. contains 2.86×10^{-7} mole.

the conditions already described. A marked decrease in the binding is obtained when the ϵ-amino positive charge is eliminated by acetylation as shown by Curve 3. This result is in accord with that found by Klotz and Urquhart.[11] A change in the binding is also obtained when the guanidyl positive charge is removed from the albumin, although the extent of combination seems to be very much the same as indicated by the intercept on the $1/r$ axis. This point of intercept, which represents the reciprocal of the value of the binding capacity (n) as used previously,[3,6] is very difficult to determine with a high degree of accuracy. The amount of extrapolation necessary to obtain this value has been pointed out by Scatchard,[12] who has plotted the values r/c against r to eliminate the disadvantage of concealing deviations from the ideal laws. This procedure has been used in this Laboratory in cases in which a more reliable value for n was

TABLE VI

BINDING OF METHYL ORANGE BY ACETYLATED BOVINE SERUM ALBUMIN AT 1° IN PHOSPHATE BUFFER, $\Gamma/2 = 0.2$, pH 7.6

Equil. concn. without prot., m./l. × 10^5	Equil. concn. with prot., m./l. × 10^5	Moles bound anion × 10^7	Moles bound anion per mole prot.,[a] r	$1/r$	$1/c$ × 10^{-4}
4.10	3.10	1.50	1.05	0.95	3.23
3.55	2.63	1.38	0.97	1.04	3.80
2.95	2.11	1.26	.88	1.14	4.75
2.38	1.68	1.05	.74	1.36	5.90
1.77	1.21	0.84	.59	1.71	8.25
1.16	0.80	0.54	.38	2.65	12.50
0.60	0.40	0.30	.21	4.76	25.00

[a] 5 ml. of protein solution contains 1.43×10^{-7} mole.

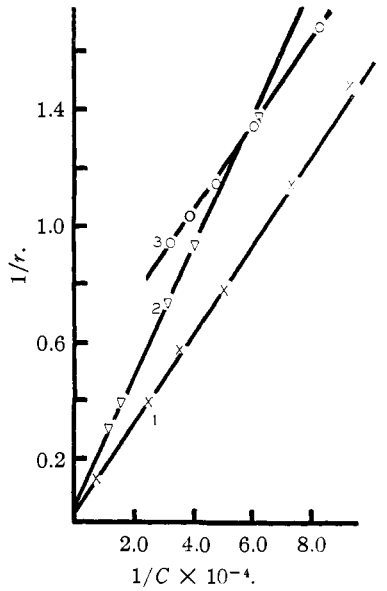

Fig. 6.—Binding data for methyl orange and native albumin (Curve 1), formaldehyde treated albumin (Curve 2), acetylated albumin (Curve 3).

(11) I. M. Klotz and Jean M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

(12) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 573 (1949).

desired. At any rate, the results on methyl orange binding suggest the essential nature of the ϵ-amino positive charge of the protein. The effect obtained in the case of formaldehyde treated albumin may indicate an influence of the guanidinium groups suggesting either the proximity of the two positive centers or the possibility that a small number of the guanidyl positive charges also act as binding centers. Another possible explanation is that the decrease in binding is due to an electrostatic repulsion of anions caused by the increase in negativity of the proteins which resulted when some of the positive charges were removed by the modification procedures. This net protein charge factor will be discussed below.

In Fig. 7 are summarized the binding data for 2,4-dichlorophenolate (Curves 1 and 2) and *o*-nitrophenolate (Curves 3 and 4). In these cases the removal of the ϵ-amino positive charges had very little effect on the extent of binding. The study of the interaction of *o*-nitrophenolate with formaldehyde-treated albumin showed no detectable combination within the limits of sensitivity of the equilibrium-dialysis method. The interaction of 2,4-dichlorophenolate and formaldehyde treated albumin was not studied, but conclusions can be drawn from the fact that removal of ϵ-amino positive charges has very little effect on the binding of this anion with bovine serum albumin. Since the ϵ-ammonium and guanidyl groups are the only contributors of positive charge at the *p*H of the experiments, by elimination it can be concluded that, as in the case of *o*-nitrophenolate, the guanidyl group is the important binding site in the binding of 2,4-dichlorophenolate. The effect exhibited by acetylated protein again may be due to the proximity of the two types of positive charges under investigation or to the protein net charge factor which is discussed elsewhere. However, because of the difficulty of assigning accurate values of n to the anions, the possibility of the involvement of a small number of ϵ-ammonium groups cannot be eliminated entirely.

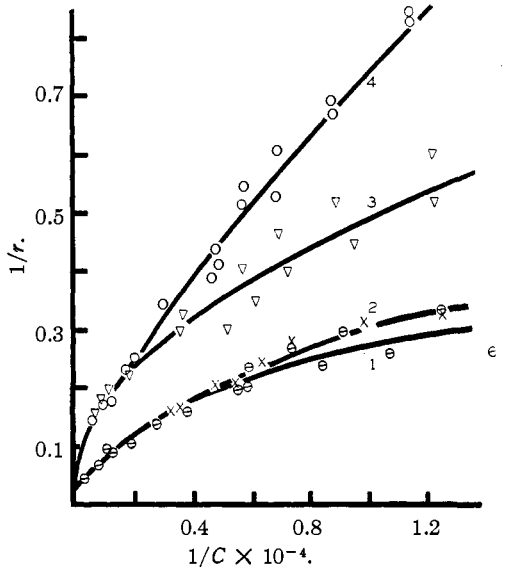

Fig. 7.—Binding data for 2,4-dichlorophenolate and *o*-nitrophenolate: curves 1 and 2 refer to 2,4-dichlorophenolate binding with native albumin and acetylated albumin respectively; curves 3 and 4 refer to *o*-nitrophenolate binding with native albumin and acetylated albumin, respectively.

The results of the combination of 2,4,6-trinitrophenolate (picrate) and *p*-nitrophenolate with albumin and modified albumins are shown in the curves in Fig. 8. Within the limits of the sensitivity of the experiment acetylated albumin showed no combination with *p*-nitrophenolate and therefore is not presented in the graph. The study of the binding of *m*-nitrophenolate by acetylated albumin showed a 92% decrease in extent of combination when compared to the values reported previously for the binding by native serum albumin.[6] The data are not graphically presented since all values of r were almost constant between 1.5 and 2 throughout the anion concentration range of 8 to 35 × 10^{-4} M. In direct contrast, acetylation had a very small effect on the combination of picrate. The treatment of the albumin with formaldehyde as described resulted in a great lowering of combination in the case of picrate, but this treatment had a small effect on the extent of combination of *p*-nitrophenolate as compared to the effect obtained by the acetylation treatment. The interaction of *m*-nitrophenolate and formaldehyde treated albumin has not been studied. The ϵ-ammonium groups seem to be necessary for the interaction of *m*- and *p*-nitrophenolate and bovine serum albumin. The effect exhibited by formaldehyde treated albumin in the case of *p*-nitrophenolate may be interpreted to mean that the guanidyl groups may contribute slightly to the

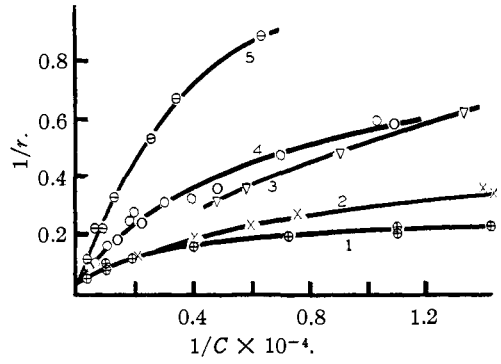

Fig. 8.—Binding data for picrate and *p*-nitrophenolate: curves 1, 2 and 3 refer to picrate binding with native albumin, acetylated albumin and formaldehyde treated albumin, respectively; curves 4 and 5 refer to *p*-nitrophenolate binding with native albumin and formaldehyde treated albumin, respectively.

total number of binding sites, that the two positive charges are close enough to each other to exert some effect on the binding constants, or that the decrease in binding is due entirely or in part to the net charge factor discussed below. In the case of the picrate and albumin interaction the guanidyl groups are greatly involved. The removal of the guanidyl positive charge did not completely prevent the picrate binding. This may be due to the incomplete formaldehyde reaction or to the possibility of the direct involvement of a small number of ϵ-ammonium groups. Perhaps the interaction of albumin and ortho substituted nitrophenolates can be clarified somewhat by a study of these with a guanidinated albumin. This line of study is now in progress in this laboratory.

It has been brought to the attention of the author that differences in the net protein charge both between native and modified proteins and between acetylated and formaldehyde treated proteins would reduce the binding by modified proteins due to an electrostatic repulsion caused by the increase in negative charge resulting from the modification of the albumin. This effect would be greater for the acetylated protein.

In this connection, however, it must be pointed out that results obtained by Klotz and Walker[13] and Klotz and Urquhart[14] showed that binding of methyl orange by serum albumin remained constant throughout the *p*H range of 5 to 9. Results previously reported by this laboratory[6] showed that the binding of picrate by serum albumin was not altered by increasing the *p*H from 7.6 to 8.2.

Since the net charge effect should be greater for acetylated albumin, it will not alter the main conclusions drawn from the results of *o*-nitrophenolate, 2,4-dichlorophenolate and picrate in view of the fact that the binding of these compounds was decreased to a very slight degree by acetylation. A correction due to net charge effect would reduce this small change produced by the removal of the lysine positive charges.

The conclusions concerning the main sites bound by *m*- and *p*-nitrophenolate may be questioned if the net protein charge effect is great enough to account for the almost complete lack of binding by acetylated albumin. The exact amount of correction required can only be determined by binding experiments performed under conditions where the proteins carry the same net charge. A program of this kind is now being contemplated in this laboratory.

Simple Theory.—The law of mass action has been applied by von Muralt[15] and Klotz[16] to the case of multiple combination resulting in the relation, shown below in equation (1), between r and c under conditions that all the binding sites have the same intrinsic association constant K and that, aside from the statistical factor, the free energy of binding to any particular site is independent of binding to other sites.

$$1/r = 1/Knc + 1/n \qquad (1)$$

The value of K depends on the nature of the protein and the anion and n represents the average maximum number of sites per protein molecule. According to the above equation a plot of $1/r$ against $1/c$ will give a straight line. From the slope of this line and its intercept with the $1/r$ axis, the values of K and n can be obtained. This procedure was used in previous work[6] in an investigation of binding of aromatic carboxylates and nitrophenolates with bovine serum albumin. The binding studies of some nitrophenolates have been carried further under conditions of higher anion concentrations as suggested by Karush and Sonenberg.[7] As can be seen in Figs. 7 and 8, the binding of these nitrophenolates by bovine serum albumin does not satisfy the conditions noted above for the simple theory. The experimental points deviate from a straight line with decrease in $1/c$, *i. e.*, with higher concentration of free anion. The data on the binding of methyl orange agree with the results reported by Klotz, Walker and Pivan[3] and satisfy the conditions required by the simple theory of the law of mass action.

The deviation from linearity obtained with nitrophenolates is similar to that obtained with homologous alkyl sulfates by Karush and Sonenberg[7] who introduced an heterogeneity theory to explain their results. Our data was treated in the same manner and the results with a brief outline of the theory is given below.

Heterogeneity Theory.—In deriving equations based on the heterogeneity theory, Karush and Sonenberg took as a model a protein molecule with a large number of binding sites which combine with small ions or molecules independently of each other. Their derivations finally led to equation (2) below

$$n/r = 1/(1 - f(c)) \qquad (2)$$

where $f(c)$ is given by equation (3).

$$f(c) = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 c e^{\alpha\sigma}} \, d\alpha \qquad (3)$$

The integral in (3) can be evaluated numerically for the computation of $f(c)$ by using Weddles rule.[17]

The application of equation (2) to experimental binding data was done by the method described by Karush and Sonenberg, but the curves were extended to give the range of $1/K_0c$ that fits the present experimental data. These curves are shown in Fig. 9.

(13) I. M. Klotz and F. M. Walker, THIS JOURNAL, **69**, 1609 (1947).

(14) I. M. Klotz and Jean M. Urquhart, *J. Phys. Coll. Chem.*, **53**, 100 (1949).

(15) A. L. von Muralt, THIS JOURNAL, **52**, 3518 (1930).

(16) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).

(17) H. Margenau and G. M. Murphy, "The Mathematics of Physics and Chemistry," D. Van Nostrand Co., New York, N. Y., 1943, Chap. 13.

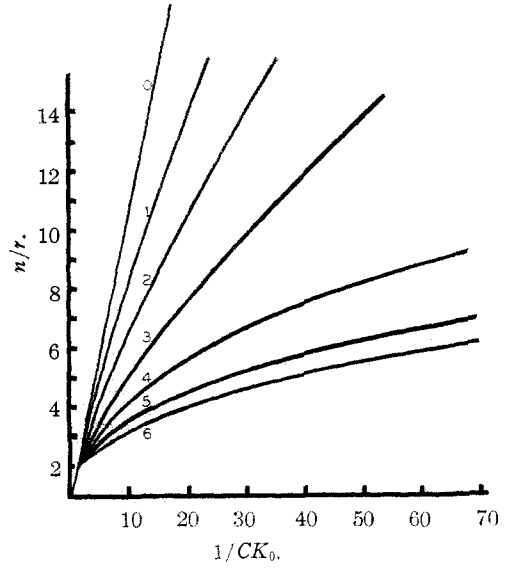

Fig. 9.—Theoretical binding curves for σ = 0 to 6 for range of very low binding.

By choosing the proper curves in Fig. 9, the theoretical curves shown as solid lines in Fig. 10 have been obtained using n equal to 25. The values of K_0 and σ employed are listed in Table VII.

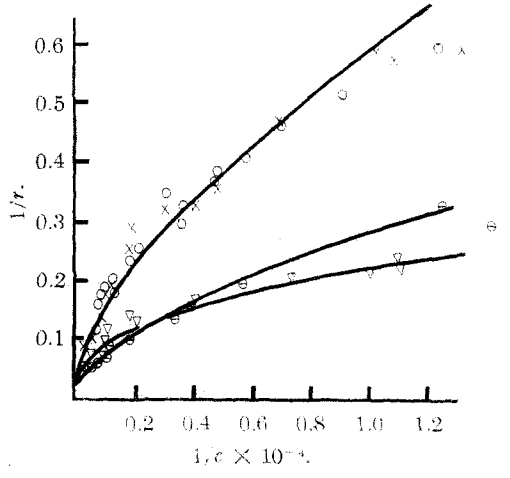

Fig. 10.—Binding data for picrate (∇), 2,4-dichlorophenolate (⊖), o-nitrophenolate (×), and p-nitrophenolate (O).

An interesting point brought out by this approach is the similarity of the values of σ for

TABLE VII

CONSTANTS AND FREE ENERGY DATA FOR BINDING OF SOME PHENOLATES BY BOVINE SERUM ALBUMIN

Anion	σ	$K_0 \times 10^{-2}$	$-\Delta F^\circ$, cal./mole
p-Nitrophenolate	2.75	2	2880
o-Nitrophenolate	2.75	2	2880
2,4-Dichlorophenolate	1.5	10	3756
Picrate	4.0	5	3380

o-nitrophenolate and p-nitrophenolate although there is good evidence to indicate that these anions react with different positive centers on the albumin molecule. On the other hand the interactions of different anions involving the same types of binding centers display a large variation in heterogeneity as is evident from the values of σ and of ΔF° for the binding processes of 2,4-dichlorophenolate and picrate.

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Summary

A comparative study has been made of the interactions of some nitrophenolates, 2,4-dichlorophenolate and methyl orange with native and modified bovine serum albumin. The protein was modified by acetylation of the ϵ-ammonium groups of the lysine residues and by the treatment of the guanidinium groups of the arginine residues with formaldehyde at pH 11.

The spectroscopic behavior of o-, p- and m-nitrophenolate, picrate, and methyl orange showed marked changes in the presence of native albumin. These changes were modified when acetylated or formaldehyde treated albumin was used instead of the native albumin. The correlation between the modification of spectral changes and the binding sites on the protein molecule was reported.

The results of quantitative studies by the dialysis-equilibrium method leading to information on the specific sites involved in the anion-albumin interactions were discussed.

The heterogeneity theory of protein binding in relation to the data obtained with picrate, p-nitrophenolate, o-nitrophenolate and 2,4-dichlorophenolate was presented and shown to fit the experimental results.

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