[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Binding of Organic Ions by Proteins. Interactions with Cations

BY IRVING M. KLOTZ, EDMUND W. GELEWITZ AND JEAN M. URQUHART

In contrast to its behavior with organic anions, serum albumin does not bind organic cations of comparable size. Its behavior with cations is similar to that of other corpuscular proteins such as bovine γ-globulin and trypsin. The relative inability of albumin to bind organic cations is consistent with the internal bonding hypothesis originally evolved to interpret the unique character of albumin in anion interactions.

Introduction

The unusual affinity of serum albumin for anions has been the subject of much recent speculation. Abundant evidence shows that the presence of cationic groups in the protein is a necessary but insufficient condition for binding. It has been suggested[1,2] that a sufficiently large number of non-polar residues, such as those of leucine or phenylalanine, must also be present. Some shortcomings of this viewpoint have been discussed recently[3] and an alternative hypothesis has been presented which assumes that within the protein molecule -OH groups form bonds with $-COO^-$ groups preferentially to bonds with $\equiv NH^+$ residues. This assumption seems warranted by the greater energy of OH···O bonds as compared to O···HN bonds[4] as well as by observations that such preferential bonding occurs in threonine[5] crystals. In a protein with approximately equivalent numbers of -OH and $-COO^-$ residues, $\equiv NH^+$ residues will not be hydrogen bonded internally and hence the macromolecule should have a strong affinity for anions. It also follows that the affinity of this same protein for organic cations should be small since the $-COO^-$ groups are "blocked" by internal -OH bonds, the breaking of which would require additional energy. In contrast, from the hypothesis of Davis[1] and of Luck,[2] the non-polar residues should assist the binding of organic cations at anionic sites about as much as they are assumed to assist the binding of organic anions at cationic sites. It has seemed of interest, therefore, to obtain quantitative binding data for some representative organic cations.

Experimental

Extent of binding was measured by the differential dialysis technique described in detail previously.[6] Optical absorption was used for analysis of the colored cations.

Streptomycin analyses were based on observations of Fried and Titus.[7] An aliquot portion of solution outside the dialysis bag was mixed with an equal quantity of 0.2 *M* NaOH. The mixture was heated in boiling water seven minutes. The absorption of light at 325 mμ by the cooled solution was used as a measure of streptomycin concentration.

Optical densities were determined in the Beckman quartz spectrophotometer with one-cm. cells. Molecular extinction coefficients, ε, were calculated from the equation

$$\log I_0/I = \epsilon cd$$

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

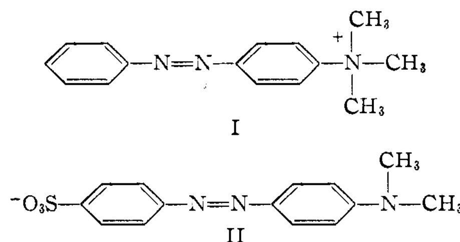
p-Azobenzenetrimethylammonium iodide was prepared by Mr. S. Preis from dimethylaminoazobenzene and excess methyl iodide dissolved in absolute ethanol; m.p. 185–186°; lit.[8,9] 185–186°. 1,1′-Diethyl-2,2′-cyanine chloride and 1-(*p*-dimethylaminostyryl)-isoquinoline methochloride were kindly given by Dr. L. G. S. Brooker of the Kodak Research Laboratories. Streptomycin, as the calcium chloride complex, was obtained from Merck and Co., Inc., through the courtesy of Dr. T. J. Webb. The neutral red was a commercial sample.

The bovine plasma proteins, γ-globulin (fraction II) and crystallized albumin, and trypsin were purchased from Armour and Company. Water contents were determined by drying separate portions at 110°.

Buffers were prepared from reagent grade materials.

Results and Discussion

Interactions with Azobenzenetrimethylammonium Ion.—In view of the extensive work on methyl orange (II), a similar cation, *p*-azobenzenetrimethylammonium ion (I), was studied first.



Dialysis–equilibrium studies (Table I) with *p*-azobenzenetrimethylammonium iodide, carried out first in phosphate buffer at *p*H 6.88, show negligible binding by bovine serum albumin (2.9×10^{-5} *M*). Over a range of cation concentration from 0.6

TABLE I

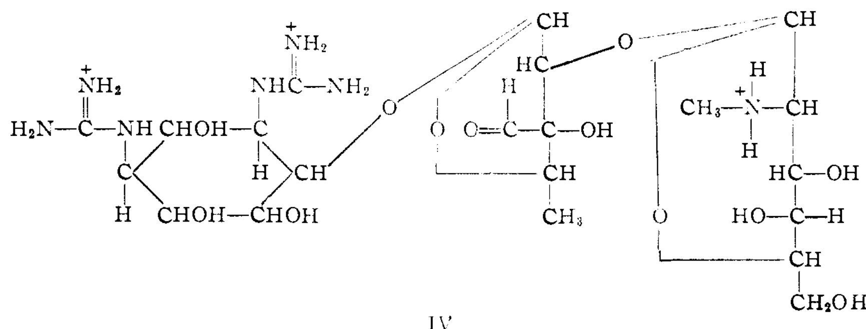
BINDING OF *p*-AZOBENZENETRIMETHLYLAMMONIUM ION BY BOVINE SERUM ALBUMIN

0.2% albumin, 0°

Concn. of cation in equilibrium with protein, $10^{+5} \times M$	Concn. of cation in absence of protein, $10^{+5} \times M$	$r = \frac{\text{moles bound cation}}{\text{moles total protein}}$
*p*H 6.88, phosphate buffer, ionic strength 0.132		
0.622	0.632	0.01
1.958	1.975	.02
3.13	3.14	.01
3.26	3.28	.02
*p*H 9.2, glycine buffer, ionic strength 0.100		
0.598	0.628	0.03
1.07	1.11	.04
1.96	1.96	.00
3.21	3.23	.02

(1) B. D. Davis, *Am. Scientist*, **34**, 611 (1946).
(2) J. M. Luck, *Discussions Faraday Soc.*, **6**, 44 (1949).
(3) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).
(4) L. Pauling, "The Nature of the Chemical Bond," 2nd edition, Cornell University Press, Ithaca, N. Y., 1945, pp. 333–334.
(5) D. P. Shoemaker, J. Donohue, V. Schomaker and R. B. Corey, THIS JOURNAL, **72**, 2328 (1950).
(6) I. M. Klotz, F. M. Walker and R. B. Pivan, *ibid.*, **68**, 1486 (1946).
(7) J. Fried and E. Titus, *ibid.*, **70**, 3615 (1948).
(8) D. Vorländer and E. Mumme, *Ber.*, **36**, 1486 (1903).
(9) A. Pongratz, G. Markgraf and E. Mayer-Pitsch, *ibid.*, **71**, 1287 (1938).

$\times 10^{-5}$ to 3.3×10^{-5} *M* the number of moles of bound dye per mole of total protein (*r*) is about 0.01. Under comparable conditions, methyl orange anions exhibit *r* values ranging from near 0.5 to nearly 2.



IV

This contrast is particularly striking from the point of view of net electrostatic charge. Since serum albumin has a substantial negative charge[10] at *p*H 6.9, it should repel an anion and attract a cation. The contrary affinities actually observed demonstrate the secondary significance of net protein charge.

This relative insignificance is further emphasized by the results of binding experiments carried out in a glycine buffer at *p*H 9. From Tanford's[11] titration data, it can be estimated that the negative charge on an albumin molecule increases by more than 15 units as the *p*H is increased from 6.9 to 9. The high *p*H should therefore favor cation binding, just as low *p*H's facilitate anion binding.[3,12] Nevertheless, no appreciable binding of the cation is observed at *p*H 9 either (Table I).

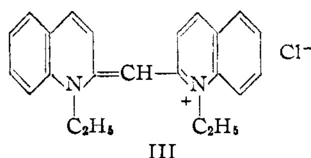
Spectra of the cation in the presence of albumin confirm the results of the dialysis experiments. Neither the wave lengths nor extinction coefficients of the absorption peaks are modified appreciably even at a protein concentration as high as 1.5% (Table II).

TABLE II

SPECTRAL CONSTANTS OF *p*-AZOBENZENETRIMETHYLAMMONIUM ION

Concn. of cation, mole/liter	Medium	Wave lengths of maxima, mμ A	B	Molecular extinction coefficients A	B
3×10^{-5}	Water	315	425	16,400	1000
3×10^{-5}	Phosphate buffer *p*H 6.88	316		16,470	
3×10^{-5}	Buffer +0.15% albumin	316		16,330	
3×10^{-4}	Buffer		426		1033
3×10^{-4}	Buffer +0.15% albumin		426		1033
3×10^{-4}	Buffer +1.5% albumin		426		1010

Interactions with Other Cations.—Experiments with other types of organic cations lead to essentially the same conclusions. The results of typical dialyses with 1,1′-diethyl-2,2′-cyanine chloride (III) (Table III) and with streptomycin



III

(IV) (Table IV) show no binding by albumin.[13]

TABLE III

BINDING OF DIETHYLCYANINE ION BY PROTEINS

0°, 0.2% protein, phosphate buffer

Concn. of cation in equilibrium with protein, $10^{+5} \times M$	Concn. of cation in absence of protein, $10^{+5} \times M$	$r = \frac{\text{moles bound cation}}{\text{moles total protein}}$
Bovine serum albumin, *p*H 5.8		
0.903	0.899	0.00
2.71	2.69	.00
3.61	3.61	.00
Bovine serum globulin, *p*H 5.8		
0.350	0.360	0.01
1.60	1.60	.00
Trypsin, *p*H 7.8		
0.240	0.238	0.00
0.550	0.560	.003
1.22	1.235	.005

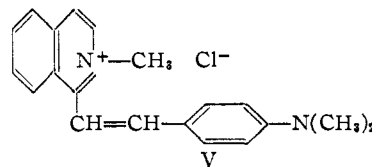
TABLE IV

BINDING OF STREPTOMYCIN BY PROTEINS

0°, 3% protein, *p*H 6.6, citrate buffer, ionic strength 0.30

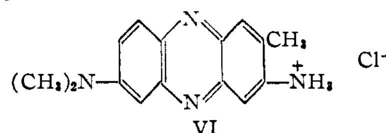
Concn. of cation in equilibrium with protein, $10^{+5} \times M$	Concn. of cation in absence of protein, $10^{+5} \times M$	$r = \frac{\text{moles bound cation}}{\text{moles total protein}}$
Bovine serum albumin		
2.06	2.12	0.003
3.81	3.80	.00
5.50	5.50	.00
9.2	9.2	.00
Bovine serum globulin		
1.21	1.25	0.004
1.90	2.05	.016
5.70	5.86	.017
8.24	8.24	.00

Spectra of 1,1′-diethyl-2,2′-cyanine chloride (*p*H 5.8) confirm the results from dialysis. Spectrophotometric examination indicates that 1-(*p*-dimethylaminostyryl)-isoquinoline methochloride (V) at *p*H 4 and neutral red (VI) at *p*H 5.7 are not



V

bound by albumin.



VI

(10) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, 68, 2320 (1946).

(11) C. Tanford, *ibid.*, 72, 441 (1950).

(12) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, 72, 535, 540 (1950).

(13) These results disagree with the conclusions of S. Berkman, R. D. Housewright and R. J. Henry, *J. Immunol.*, 61, 349 (1949). However, in their experiments no corrections were made for Donnan equilibria. Those experiments which were carried out in the presence of salt point very strongly toward the absence of streptomycin–albumin complexes.

Less direct methods also give no indication of interactions of albumin with cations. The cloud-point technique[2] indicates the absence of complexes with aliphatic amines, although interpretation of the data is complicated by factors other than binding. The pH displacement method[14] reveals a similarity in behavior of the quaternary amines tetrapropylammonium iodide and trimethylphenylammonium iodide, and sodium iodide. Since there is strong evidence[12] that sodium ion is not bound by albumin, it seems reasonable to interpret the pH behavior of the quaternary ammonium ions in a like fashion.

Thus it is clear that organic cations of a wide variety of structures do not form complexes with serum albumin with an affinity even approaching that of complexes with anions of similar structure.

Comparison of Proteins.—The binding behavior of albumin is markedly different from that of the fibrous protein wool. Steinhardt and Zaiser[15] have shown that quaternary ammonium ions affect the titration curves of wool with bases in a manner analogous to the effects of anions on acid titrations. Thus both anions and cations of about equal size seem to be bound by wool.

The behavior of albumin toward organic cations is as predicted by the postulate[3] that residues with -OH groups interact preferentially with $-COO^-$ side chains. Because of this internal bonding, an organic cation must have a much stronger interaction energy to be bound at a $-COO^-$ site than an organic anion requires to be bound at an $\equiv NH^+$ site. Among molecules of similar size and structure, the anions are therefore bound much more strongly than cations.

From the hypothesis of preferential hydroxyl–carboxyl internal bonding it also follows that the unique character of serum albumin in anion–protein interactions should disappear in cation–protein interactions. Among the proteins previously compared[8] in anion affinities, since all except albumin have an excess of -OH groups over carboxylic side chains all should have little affinity for organic cations. The globular proteins bovine γ-globulin and trypsin behave as predicted, neither binding the diethylcyanine ion (Table III). Similarly γ-globulin shows no interaction with streptomycin (Table IV).

Thus in interactions with organic cations albumin loses its unique position and acts as a typical corpuscular protein.

Acknowledgment.—This investigation was supported in part by research grants from the National Institutes of Health, Public Health Service, and from the Office of Naval Research (Project Number NR 121-054).

EVANSTON, ILL. RECEIVED APRIL 18, 1951

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(15) J. Steinhardt and E. M. Zaiser, *J. Biol. Chem.*, **183**, 789 (1950).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Effect of pH on the Combination of Serum Albumin with Metals[1]

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A polarographic study has been made of the effect of pH upon the interaction between a number of metals and bovine serum albumin. It is concluded that the principal sites on the protein molecule responsible for metal binding are the imidazole groups. Approximate values of the logarithms of the intrinsic association constants with these groups are: Cu^{++} (in 0.15 M KNO_3) 3.7, Zn^{++} (in 0.15 M KCl) 2.9, Cd^{++} (in 0.15 M KCl), 2.8, Pb^{++} (in 0.15 M KNO_3) < 2.3, Tl^+ (in 0.15 M KCl) < 0. Weak binding also occurs at carboxyl groups. The extent of binding at these sites is determined largely by the competitive effect of other anions present in solution.

It is well-known that the limiting polarographic current due to the reduction of a metallic ion (or other reducible substance) may be considerably decreased by the presence of proteins.[2,3] Previous work in this Laboratory[4] has shown that this decrease is due to complex formation between the metallic ion and the protein; while a metal ion involved in such a complex may still be reduced, the resulting current will be smaller because of the smaller diffusion coefficient of a metal ion bound to a protein molecule, and also because of the fact that the reduction itself may proceed slowly.

Work is currently under way in this Laboratory to develop a method for obtaining thermodynamic constants for protein–metal interaction from this decrease in the polarographic current. The present paper, however, describes how semi-quantitative information on metal–protein complexes can be obtained with great rapidity from the polarographic current depression. The information obtained is similar to that which can be gained from the observation of shifts in absorption spectrum peaks. The work reported is a study of complex formation between serum albumin and cadmium, zinc, lead, copper and thallium, and of the effect of pH upon the extent of interaction.

Experimental

Polarographic currents were measured on a Sargent model XXI polarograph. The capillary used has a flow rate of 2.65 mg. of mercury per sec., with a drop time varying between 3.6 and 4.0 sec., depending on the solution used. The cell designed for this work and the technique for oxygen removal have been described elsewhere.[5] Polarographic currents were measured by the extrapolation method.

Armour crystalline bovine serum albumin was used, and its concentration in stock solutions was determined by means of ultraviolet light absorption at 280 mμ wave

(1) Presented at the XIIth International Congress of Pure and Applied Chemistry, New York, N. Y., September, 1951.

(2) I. M. Kolthoff and J. J. Lingane, "Polarography," Interscience Publishers, Inc., New York, N. Y., 1941, p. 121.

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