[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Interactions of Some Neutral Organic Molecules with Proteins

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To complement earlier investigations of the interactions of proteins with organic anions and cations, the binding of some uncharged molecules has been investigated. Complex formation with bovine and with human albumin becomes significant near *p*H 7 and continues to increase to *p*H 9. This trend provides further evidence of configurational changes in the albumin molecule as *p*H is varied. Experiments with serum albumin in which lysine, tyrosine and carboxyl residues have been modified indicate that binding of aminoazobenzene involves a hydrogen bond between a side chain of the protein and an electron pair of the uncharged molecule. Anions and neutral molecules may compete for common sites of serum albumin. Likewise, the unique behavior of this protein in anion interactions is carried over to neutral molecules.

Introduction

Extensive studies have been made recently of the binding of anions by proteins,[1-3] particularly by serum albumin. Some investigations have also been carried out of relative affinities of proteins for organic cations[4] as compared to anions. Many of the observations reported have raised questions with respect to molecular changes in serum albumin which might be clarified by an examination of interactions with uncharged organic molecules. The results described in this paper indicate that neutral molecules may compete with anions for sites on albumin, and that the availability of binding sites on the protein, and hence its configuration, is markedly dependent on its charge.

Experimental

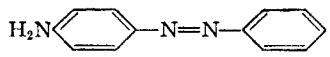
Reagents.—*p*-Aminoazobenzene (Eastman Kodak Co. "white label") was recrystallized three times from a methanol–water mixture. Analysis by titanous chloride reduction of the azo group[5] indicated a purity of 96.3%. The chrysoidin was a commercial sample (Grübler and Co.) and was used without further purification. Crystalline bovine serum albumin and bovine γ-globulin were purchased from Armour and Co. Human serum albumin was obtained from Drs. P. H. Bell and R. O. Roblin, Jr., of the American Cyanamid Co. It had been prepared from contaminated plasma by standard blood bank methods and was 97–99% pure as estimated by electrophoretic methods.[6] Bovine albumin was acetylated according to the procedure of Fraenkel-Conrat, Bean and Lineweaver.[7] Analyses for free ε-amino groups[8] showed that 60 ± 10% of these groups had been acetylated. Iodinated bovine albumin was prepared by Dr. W. L. Riedeman, following the procedure of Hughes and Straessle.[9] Absorption of light of 312 mμ by this protein corresponded to 9 diiodotyrosine residues. Methoxylated bovine albumin, in which free carboxyl groups have been converted to the methyl ester, was prepared by Dr. W. W. Weber according to the method of Fraenkel-Conrat and Olcott.[10] Methoxyl analyses based on the general technique described by Elek[11] indicated that approximately 70% of the carboxyl groups in the protein had been esterified. A sample of guanidinated human albumin[12] was obtained through the courtesy of Professors E. J. Cohn and W. L. Hughes, Jr., of Harvard University. It had been prepared from crystallized human albumin, lot 179-5x, and had 34 modified ε-amino groups.

The inorganic salts were reagent grade. The buffers used were as follows: *p*H 4.7, 0.1 *M* acetate; *p*H 5.8 and 6.9, 0.1 *M* phosphate; *p*H 9, 0.05 *M* borate in chrysoidin experiments, 0.3 *M* glycine plus 0.1 *M* glycinate in aminoazobenzene experiments.

Dialysis Experiments.—The extent of binding of each of the substances studied was measured by the differential dialysis technique described previously.[13] Experiments were carried out with mechanical shaking for an 18-hour period at 0.0 ± 0.1° and at 25.00 ± 0.05°, respectively. Preliminary studies indicated that equilibrium was reached in a period of about six hours. The protein concentration was 0.2%.

Results and Discussion

The very first experiments showed that bovine serum albumin binds the neutral dye aminoazobenzene (I), though with an affinity somewhat



I

smaller than that for the anionic dye methyl orange. Representative data are illustrated in Fig. 1 in terms of the usual coördinates,[2] *r*, the moles of bound dye per mole total protein, *versus* the logarithm of (A), the moles of free dye.

Competition with Anions.—In connection with the interpretation of *p*H effects[14,15] on anion binding it seemed desirable at the outset to examine the possibility that anions and neutral molecules may compete for common sites on albumin. Addition of sodium chloride to a concentration of 0.1 *M* produced no significant displacement of aminoazobenzene (Fig. 1). Sodium thiocyanate at the same concentration, on the other hand, reduced the uptake of the neutral molecule by a significant amount (Fig. 1). Clearly the SCN^- ion displaced aminoazobenzene. The greater displacing ability of thiocyanate as compared to chloride is in line with the relative affinity of serum albumin for these two anions.[16]

Effect of *p*H.—Binding of aminoazobenzene by albumin increases substantially as the *p*H is raised from 6.9 to 9.2 (compare Figs. 1 and 2). This

(1) F. Karush, *J. Phys. Chem.*, **56**, 70 (1952).

(2) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, THIS JOURNAL, **74**, 202 (1952).

(3) G. Scatchard, *Am. Scientist*, **40**, 61 (1952).

(4) I. M. Klotz, E. W. Gelewitz and J. M. Urquhart, THIS JOURNAL, **74**, 209 (1952).

(5) S. Siggia, "Quantitative Organic Analysis via Functional Groups," John Wiley and Sons, Inc., New York, N. Y., 1949, pp. 82–84.

(6) P. H. Bell, private communication.

(7) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1945).

(8) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 235 (1936).

(9) W. L. Hughes, Jr., and R. Straessle, THIS JOURNAL, **72**, 452 (1950).

(10) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(11) A. Elek, *Ind. Eng. Chem., Anal. Ed.*, **11**, 174 (1939).

(12) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, THIS JOURNAL, **71**, 2476 (1949).

(13) I. M. Klotz, F. M. Walker and R. B. Pivan, *ibid.*, **68**, 1486 (1946).

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

(15) F. Karush, THIS JOURNAL, **73**, 1246 (1951).

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

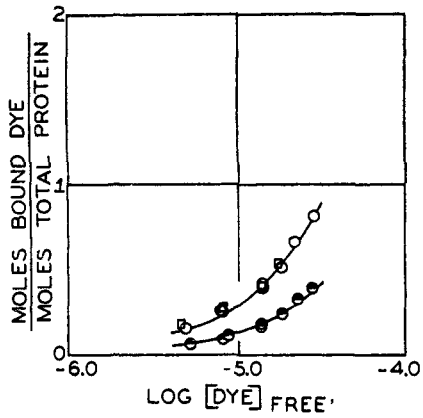


Fig. 1.—Binding of *p*-aminoazobenzene by bovine serum albumin at 25° in phosphate buffer at *p*H 6.9: O, dye alone; □, dye, in presence of 0.1 *M* NaCl; ◒, dye in presence of 0.1 *M* NaSCN.

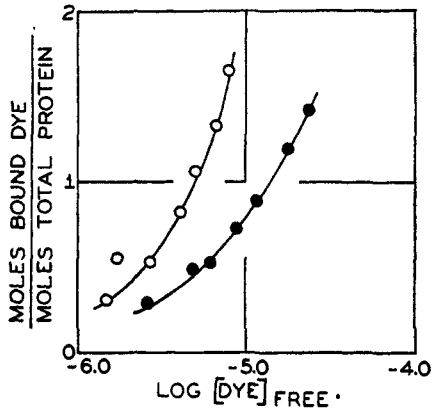
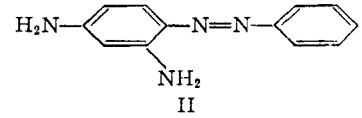


Fig. 2.—Binding of *p*-aminoazobenzene by bovine serum albumin in glycinate buffer at *p*H 9.2: O, 0°; ●, 25°.

trend is of interest in several connections. First it offers an explanation for the different results observed by Karush[15] and by Klotz and Urquhart[14] in their investigations of the effect of *p*H on anion binding. In the former's experiments binding of methyl orange was found to decrease somewhat as the *p*H was increased from 7.5 to 9; in contrast the latter investigators found an increase over the same *p*H range. In the experiments of Karush the aqueous dye–protein solution was equilibrated with an immiscible organic phase containing hexanol. That hexanol competes with the anion was pointed out by the author.[15] The present experiments show furthermore that neutral molecules are bound more strongly as the *p*H is increased. If neutral molecules and anions continue to compete for some common sites, it is clear that the former should be more effective in displacing the latter as the protein acquires a more negative charge. Despite this competition with hexanol, methyl orange binding decreases only slightly with increased *p*H, much less than would be anticipated from the increased negative charge on the protein. A correction for the competitive effect of hexanol would reduce even further this apparent decrease in binding of the anion reported by Karush. Thus it seems very likely that binding of methyl orange actually increases in the *p*H region 7 to 9 even in the absence of buffers, in accord with reported observations in the presence of buffers.[14]

Similar observations have been made with chrysoidin (II) over an even wider range of *p*H



II

(Fig. 3). It seems apparent, therefore, that new binding sites become available as albumin acquires an increasingly negative charge. The same conclusion has been reached already from studies with anions.[2] These new sites at higher *p*H's make it possible for anion and neutral molecule to actually reverse the order in which they are removed from the aqueous medium by the protein. Thus at *p*H 6.9 more methyl orange is bound to albumin than is aminoazobenzene; at *p*H 9, however, substantially greater quantities of aminoazobenzene are held by albumin.

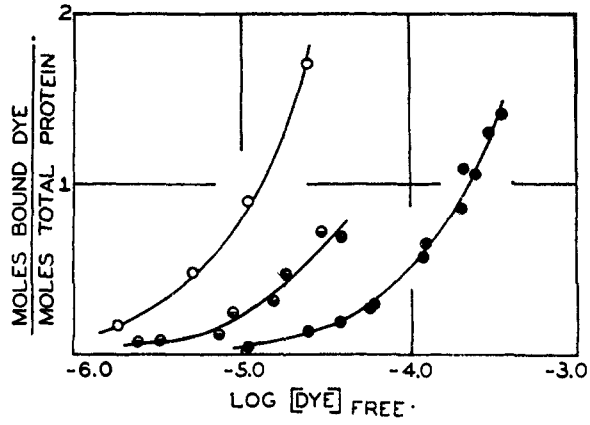


Fig. 3.—Binding of chrysoidin by bovine serum albumin at 0°: ●, in acetate buffer, *p*H 4.7; ◒, in phosphate buffer, *p*H 5.8; O, in borate buffer, *p*H 9.

Effect of Temperature.—In striking contrast to albumin–anion interactions, the binding of aminoazobenzene shows a marked temperature dependence (Fig. 2). It is of interest that such a difference between negatively charged and neutral molecules should appear in these protein interactions, since a small temperature coefficient is characteristic of interactions between oppositely charged species in simple systems also.

For purposes of quantitative comparison, thermodynamic properties of albumin complexes of aminoazobenzene have been evaluated by methods described previously[13,14,17] and are summarized in Table I. Particularly noteworthy is the relatively large enthalpy change in the binding of the neutral molecule as contrasted to the anion methyl orange.[17]

Effect of Chemical Modification of Albumin.—Since aminoazobenzene competes with anions in albumin binding, it has seemed likely that cationic side-chains of the protein are sites for the uptake of the neutral molecule also. This assumption has been corroborated by an observed decrease in affinity of acetylated bovine serum albumin as compared to the parent protein (Fig. 4). This

(17) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, 71, 847 (1949).

TABLE I

THERMODYNAMICS OF BINDING OF *p*-AMINOAZOBENZENE BY PROTEINS

Protein, albumin	Temp., °C.	*p*H	ΔF_1°, cal. mole^{-1}
Bovine serum	25	6.9	−6150
Bovine serum	25	9.2	−6910[a]
Bovine serum	0	9.2	−6640[a]
Acetylated bovine	25	9.2	−6540
Iodinated bovine	25	9.2	−6360
Methoxylated bovine	25	6.9	−6120
Human serum	25	6.9	−6310
Human serum	25	9.2	−7070
Guanidinated human	25	6.9	−6590

[a] These data at *p*H 9.2 lead to a value of −3700 cal. mole^{-1} for ΔH_1° and of 11 cal. mole^{-1} deg.$^{-1}$ for ΔS_1°. These figures, as well as those for ΔF_1°, refer to the formation of the first complex of albumin and aminoazobenzene.

acetylated protein had approximately half of its lysine groups blocked.

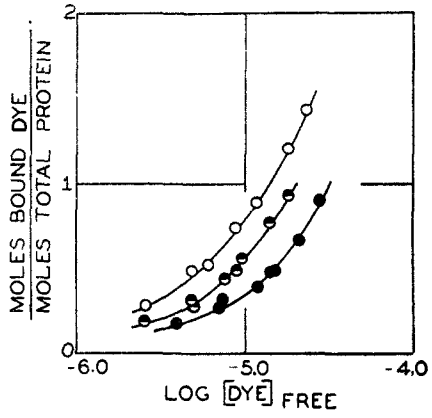


Fig. 4.—Binding of *p*-aminoazobenzene in glycinate buffer at 25° and at *p*H 9.2, by bovine serum albumin, O; by acetylated bovine serum albumin, ◐; by iodinated bovine serum albumin, ●.

Cationic side chains, however, are not the only type involved in interactions with aminoazobenzene. That such is the case became apparent first in competition experiments with thiocyanate ions. These inorganic anions can displace much more methyl orange from a protein complex than they can displace aminoazobenzene. Evidently then the neutral molecules are bound at some sites more strongly than are the anions. Experiments with iodinated bovine albumins (Fig. 4) indicate that tyrosine residues are involved in these interactions. The binding of aminoazobenzene drops 50% with this iodinated protein. This decrease is particularly striking in view of the fact that only nine diiodotyrosine residues were formed in iodoalbumin whereas over thirty lysine groups were covered in the acetylated protein. Thus the change in net charge in the latter modified protein was much greater than in the former. Evidently then the greater decrease in affinity with iodoalbumin cannot be due to a change in the over-all charge of the protein but rather to the specific involvement of tyrosine side chains in the binding of the neutral molecule.

Since lysine or tyrosine residues may participate in complex formation with aminoazobenzene, it seems likely that it is the nitrogen of the amino substituent of the neutral molecule which acts as an electron donor for hydrogen-bond formation with these side chains of the protein. This interpretation seems substantiated by the experiments with methoxylated albumin, which shows no significant change in affinity toward *p*-aminoazobenzene (Table I). Evidently the amino nitrogen does not contribute toward a hydrogen bond with a carboxyl group of the protein. It is also pertinent to point out that in interactions with a small neutral molecule the substantial change in net charge of albumin upon conversion to the methoxyl modification does not produce any significant change in affinity.

In view of these findings it was not surprising to observe little change in uptake of aminoazobenzene when human serum albumin was compared with the guanidinated protein (Table I) in which ϵ-ammonium groups of lysine have been converted to guanidinium groups. An unequivocal comparison could not be made, however, since the parent protein, Harvard lot 179-5x, from which the guanidinated sample had been prepared was not available.

Comparison of Native Proteins.—In the binding of methyl orange some remarkable differences have been observed between bovine and human albumins both with respect to the extent and nature of the interactions.[2] These differences do not seem to carry over, however, to interactions with aminoazobenzene. This neutral molecule is bound to approximately the same extent by both proteins, at *p*H 6.9 and at *p*H 9.2. Since the unusual interaction of methyl orange with human albumin has been shown to involve both polar substituents of the azobenzene nucleus of the small molecule, it is not surprising that the neutral molecule with a single substituent cannot interact simultaneously with two sites on the protein.

Like bovine albumin, human albumin shows increased affinity for aminoazobenzene as the *p*H is increased from 6.9 to 9.2 (Table I). Evidently both proteins undergo similar changes as the net charge increases. As has been described previously[2] albumin seems to swell or unfold as the *p*H rises and thereby new sites become available for interactions with small molecules.

As in anion interactions, serum albumin seems to be unique, in comparison with other proteins, in its ability to form complexes with neutral molecules. Experiments with bovine γ-globulin, at *p*H 9 as well as at 6.9, have failed to reveal any binding of aminoazobenzene. Likewise, in connection with other studies, Dr. W.-C. L. Ming has found no uptake of a neutral azopyridine molecule by pepsin. Evidently the structural pattern of albumin which establishes its unusual affinity for anions also determines its behavior toward neutral molecules.

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