by the addition of 50 ml. of ammonia water to the cooled refluxed solution followed by several chloroform extractions. The chloroform extract was then steam distilled. After all the volatile material was removed, the oily residue was dissolved in methanol, water added to cause an incipient precipitate and allowed to crystallize overnight; yield 3.0 g., recrystallized from methanol.

Isolation of Benzylacetophenone.—To the filtrate and washings of the triphenylpyridine experiment 75 ml. of ammonia water was added, the solution extracted with chloroform and the chloroform extract was steam distilled. After the chloroform and acetophenone have distilled, the benzylacetophenone steam distils more slowly and 0.8 g. appears as a slowly crystallizable oil. Upon recrystallization from dilute acetic acid brilliant platelets appear, m.p. 71° (cor.), oxime m.p. 82–84° (cor.).

Anal. Calcd. for oxime: N, 6.22. Found: N, 6.14.

Pentaphenylpyridines.—A mixture of 2 g. (0.01 mole) of desoxybenzoin, 0.55 g. (0.005 mole) of benzaldehyde, 15 g. of ammonium acetate and 30 g. of acetamide was heated gently with stirring. At a gentle reflux temperature, small bubbles developed, smelling strongly of ammonia. After 2 hours, the reaction mixture was cooled slowly, 20 cc. of methanol added and the solution heated to boiling. Upon cooling crystals appeared gradually along with an oily solid. The crystals were filtered, redissolved in glacial acetic acid and methanol added to precipitate the pentaphenylpyridine; yield 0.35 g. recrystallized from acetic acid–methanol. The 4-(3,4-methylenedioxy)-phenyl derivative was obtained in a similar manner using a ratio of 1:1.5 for the aldehyde and ketone. Recrystallized from acetic acid in fine white needles.

Acknowledgment.—The author is grateful to Professor David Davidson for the many stimulating discussions, his encouragement and the interest shown in this work.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

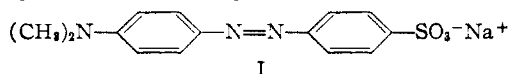
Structural Specificities in the Interactions of Some Organic Ions with Serum Albumin

BY IRVING M. KLOTZ, R. K. BURKHARD[1] AND JEAN M. URQUHART

Spectrophotometric investigations indicate that above *p*H 7 human albumin interacts in a specific fashion with anions of the structure $(CH_3)_2N-C_6H_4-N{=}N-C_6H_4-X^-$, where $-X^-$ may be $-SO_3^-$, $-CO_2^-$, $-PO_3H^-$ or $-AsO_3H^-$. If $-X^-$ is ortho, however, the specific interaction disappears. Studies with compounds containing additional substituents which block the azo or the dimethylamino nitrogen show that the latter nitrogen is involved in a linkage to the protein. Experiments with modified proteins suggest that tyrosine residues from human albumin complete this link. A bond is also formed between $-X^-$ and cationic sites of the protein. In view of the dependence of the interaction on the position of $-X^-$, it is suggested that the distance between the $(CH_3)_2N-$ and $-X^-$ substituents is the determining factor. Then at least some side chains from tyrosine residues must be about 12–13 Å. away from certain cationic loci on albumin. A method thus seems available for establishing distances between specific side chains in a protein molecule.

Introduction

Optical studies of the interactions of some azobenzene anions with bovine and human albumin, respectively, have shown[2,3] marked differences in the nature of the effects produced by these proteins. In aqueous solutions below about *p*H 7, both albumins change the spectrum (Fig. 1) of methyl orange (I) to that in organic solvents,[3] in which the



salt does not ionize appreciably. Such optical behavior is in accord with chemical evidence that the anion is bound to the albumin largely through electrostatic interaction between the $-SO_3^-$ substituent and cationic loci of the protein. Bovine albumin behaves in essentially the same manner between *p*H 7–11 also.

On the other hand, human albumin produces an effect on the spectrum of methyl orange (Fig. 1) near and above *p*H 7 markedly different than at lower *p*H's. Apparently human albumin undergoes some configurational change in a *p*H region above neutrality, and in this region an arrangement of residues becomes available which is markedly different from that in bovine albumin. To obtain some insight into these differences studies have been carried out with isomers and analogs of methyl orange and with several chemically modified proteins. It has been possible to demonstrate that both substituents on the azobenzene skeleton of methyl orange are involved in interactions with human albumin at high *p*H's. These studies indicate further that it may be possible to establish the distances between certain side chains in native albumins from an examination of optical aspects of interactions with small molecules.

Experimental

Absorption Spectra.—The absorption of light was measured with the Beckman spectrophotometer, model DU, at approximately 25°. One-cm. cells were used. Extinction coefficients, ϵ, were calculated from the equation

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

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 the solute and d the thickness of the absorption cell in centimeters.

At *p*H 9, the following extinction coefficients were calculated at the corresponding absorption maxima.

Anion	λ_{max}, mμ	ϵ
Methyl orange	465	26,800
4′-Dimethylaminoazobenzene-3-sulfonate	460	25,500
4′-Dimethylaminoazobenzene-2-sulfonate	450	21,200
Methyl red	430	21,200
4′-Dimethylaminoazobenzene-3-carboxylate	455	23,200
4′-Dimethylaminoazobenzene-4-carboxylate	465	26,400
4′-Dimethylaminoazobenzene-3-phosphonate	450	19,800
4′-Dimethylaminoazobenzene-4-phosphonate	455	21,500
4′-Dimethylaminoazobenzene-4-arsonate	460	19,400

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(2) I. M. Klotz and F. M. Walker, *J. Phys. & Colloid Chem.*, **51**, 666 (1947).

(3) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, in press.

Anion	λ_{max}, mμ	ε
Ethyl orange	475	31,500
Propyl orange	480	34,000
Butyl orange	480	33,900
4'-Dimethylamino-2,2'-dimethylazobenzene-4-sulfonate	450	22,200
4'-Hydroxyazobenzene-4-carboxylate	440	21,800

Dialysis Experiments.—The extent of binding was measured by the differential dialysis technique described in detail previously.[4] Experiments were carried out with mechanical shaking for an 18-hour period at 0.0 ± 0.1°. The protein concentration was between 0.1 and 0.2%.

Azo Compounds.—Methyl orange (I) was a commercial sample of reagent grade. Sodium 4'-dimethylaminoazobenzene-3-sulfonate (VII) and sodium 4'-dimethylaminoazobenzene-2-sulfonate (VI), were prepared essentially according to the directions of Fieser[5] with metanilic acid and orthanilic acid, respectively, being substituted for sulfanilic acid.

Methyl red (VIII) was a commercial sample but was purified by Soxhlet extraction with toluene, followed by recrystallization first from toluene and then from a pyridine–water mixture until a constant melting point was reached.

4'-Dimethylaminoazobenzene-3-carboxylic acid and 4'-dimethylaminoazobenzene-4-carboxylic acid ("meta methyl red" and "para methyl red," respectively), were prepared by a procedure[6] for the synthesis of methyl red except that *m*- and *p*-aminobenzoic acid, respectively, were coupled with dimethylaniline. The crude products were purified like methyl red.

As a further check on the purity of the dyes prepared in this manner, meta methyl red was subjected to chromatographic analysis.[8] Only a single component was eluted.

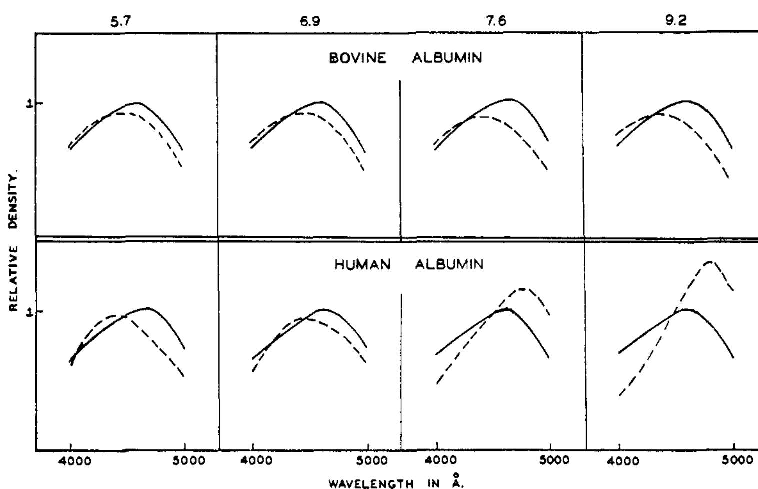


Fig. 1.—Comparison of effects of bovine albumin and of human albumin (lot 179-5x) on the spectrum of methyl orange at each of a series of *p*H's (listed at top of figure). The optical density of methyl orange alone has been set at a relative value of 1 at the maximum to facilitate comparison. Concentration of methyl orange, 1.1 × 10^{-5} to 1.2 × 10^{-5} *M;* concentration of protein, 0.20%.

PERTINENT ANALYTICAL DATA

	Melting point, °C. Obsd.	Lit.[7]	$C_{15}H_{15}O_2N_3$, % N Found	Calcd.
Meta methyl red	199–200	210	15.88	15.61
Para methyl red	271–273	...	15.85	15.61

Sodium 4'-dimethylaminoazobenzene-4-phosphonate (III) and sodium 4'-dimethylaminoazobenzene-3-phosphonate (X), were also prepared according to the procedure of Fieser[5] but with *p*-aminobenzenephosphonic acid and *m*-aminobenzenephosphonic acid, respectively, in place of sulfanilic acid. Analyses for carbon and nitrogen were consistently below theoretical, but it is probable that the impurity was merely sodium chloride.

4'-Dimethylaminoazobenzene-4-arsonic acid (IV) was a commercial sample used without further purification.

Ethyl orange, sodium 4'-diethylaminoazobenzene-4-sulfonate (XIII), was prepared[5] by coupling diazotized sulfanilic acid with diethylaniline. The crude compound was purified by Soxhlet extraction with water followed by several recrystallizations from an alcohol–water mixture. *Anal.* Calcd. for $C_{16}H_{18}O_3N_3SNa$: N, 11.8. Found: N, 11.3.

Propyl orange, 4'-dipropylaminoazobenzene-4-sulfonic acid (XIV), and butyl orange, 4'-dibutylaminoazobenzene-4-sulfonic acid (XV), were prepared[9] by coupling diazotized sulfanilic acid with the appropriate amine. In each case the crude dye was purified by three Soxhlet extractions alternately with water and toluene. After several recrystallizations from an acidic alcohol–water mixture, the observed m.p. of propyl orange was 224° (literature[10] 225°); butyl orange decomposed at 204° (literature[10,11] 198°, above 200°). *Anal.* Calcd. for $C_{20}H_{27}O_3N_3S$: N, 10.8. Found: N, 10.9.

4'-Dimethylamino-2,2'-dimethylazobenzene-4-sulfonic acid (XI) was prepared[5] by coupling diazotized 2-aminotoluene-5-sulfonic acid with N,N-dimethyl-*m*-toluidine. The aminotoluenesulfonic acid was prepared[12] by sulfonation of *o*-toluidine. The crude dye was purified like the alkyl oranges. *Anal.* Calcd. for $C_{16}H_{19}O_3N_3S$: neut. equiv., 333. Found: neut. equiv., 333 ± 6.

To determine whether the coupling took place in an unexpected position, the dye was reduced and 2-amino-5-dimethylaminotoluene was then identified by its acetyl derivative; m.p., observed, 158–158.5°; literature,[13] 158°.

o-Toluidine orange (XII), 4'-dimethylamino-3'-methylazobenzene-4-sulfonic acid, was prepared by coupling diazotized sulfanilic acid with N,N-dimethyl-*o*-toluidine. The reaction was best carried out by isolation of the diazonium salt of sulfanilic acid and coupling in glacial acetic acid. The crude dye was purified by the extraction procedures described above.

4'-Hydroxyazobenzene-4-carboxylic acid (V) and its corresponding sulfonic acid analog were obtained from Mr. S. Preis of this Laboratory.

Proteins.—The bovine serum albumin was a crystalline sample obtained from Armour and Co. The crystallized preparations[14] of human albumin labeled lot 179-5x and Decanol 10, respectively, were gifts of Professors E. J. Cohn and W. L. Hughes, Jr., of the Harvard Medical School. The former sample was crystallized with the aid of chloroform, the latter decanol. A third sample of human albumin, obtained from Drs. P. H. Bell and R. O. Roblin, Jr., of the American Cyanamid Co. and prepared from contaminated plasma by standard blood bank methods, was 97–99% pure as estimated by electrophoretic methods.[15]

Samples of guanidinated[16] and iodinated human albumin[17]

(4) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, 68, 1486 (1946).

(5) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Company, New York, N. Y., 1941, pp. 208–210.

(6) "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, pp. 374–377.

(7) A. Thiele and O. Peter, *Z. anorg. allgem. Chem.*, 173, 169 (1928).

(8) We are indebted to Dr. David Zaukelies for carrying out these experiments.

(9) W. J. Hickinbottom and E. W. Lambert, *J. Chem. Soc.*, 1383 (1939).

(10) K. H. Slotta and W. Frank, *Ber.*, 66, 104 (1933).

(11) J. Reilly and W. J. Hickinbottom, *J. Chem. Soc.*, 113, 99 (1918).

(12) M. N. Schultz and H. J. Lucas, THIS JOURNAL, 49, 298 (1927).

(13) C. Würster and C. Riedel, *Ber.*, 12, 1798 (1879).

(14) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, 69, 1753 (1947).

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

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

(17) W. L. Hughes, Jr., and R. Straessle, *ibid.*, 72, 452 (1950).

were obtained from Drs. W. L. Hughes, Jr., and H. A. Saroff of the Harvard Medical School. The guanidinated, prepared from the native protein of lot 179-5x, contained 57 guanidinated amino groups. The iodinated albumins were modifications of lot Decanol 10. The sample labeled "50% iodinated" contained sufficient iodine to convert half of the 18 tyrosine residues to diiodotyrosine, but spectra show that only 6 residues were changed. The "100% iodinated" sample contained enough iodine to convert all 18 tyrosine groups but chemical evidence[17] indicates that only about thirteen were altered.

The American Cyanamid sample and bovine albumin were acetylated[18]; analyses[19] for free ε-amino groups showed that over 80% of the groups on each albumin had been acetylated.

Buffers.—Buffers were prepared from reagent grade phosphate and tetraborate salts. Phosphate buffers were 0.10 M, borate 0.05 M.

Results and Discussion

Effect of Variation in Type of Substituent.—In connection with the unusual influence of human albumin on the spectrum of methyl orange at high pH's, the spectra (Figs. 2 and 3) of the corresponding carboxylic (II), phosphonic (III) and arsonic (IV) acids were taken in the presence of this protein at pH 9. Since the nature of the spectral shift is the same as for methyl orange, the type of anionic substituent is not important.

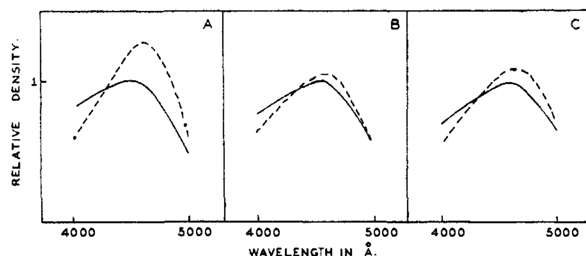
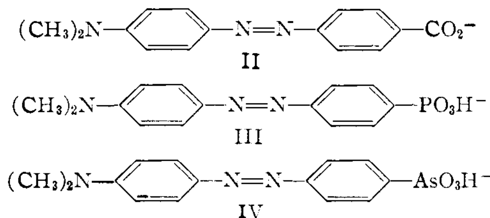


Fig. 2.—Effects of human albumin (American Cyanamid Co.) on the spectra of: A, 4′-dimethylaminoazobenzene-3-phosphonate (X) ion, 1.03 × 10^{-5} M; B, 4′-dimethylaminoazobenzene-4-phosphonate (III) ion, 2.09 × 10^{-5} M; C, 4′-dimethylaminoazobenzene-4-arsonate (IV) ion, 1.21 × 10^{-5} M; borate buffer, pH 9.2; protein concentration, 0.2%.



The optical exaltation produced with these para compounds (I to IV) is greater for the sulfonate and carboxylate than for the phosphonate and arsonate. Since extent of optical displacement is a measure of degree of binding,[20] the phosphonate and arsonate compounds are less strongly bound. At pH 9, these two compounds are doubly charged, and the electrostatic repulsion by the negatively charged albumin molecule is increased.

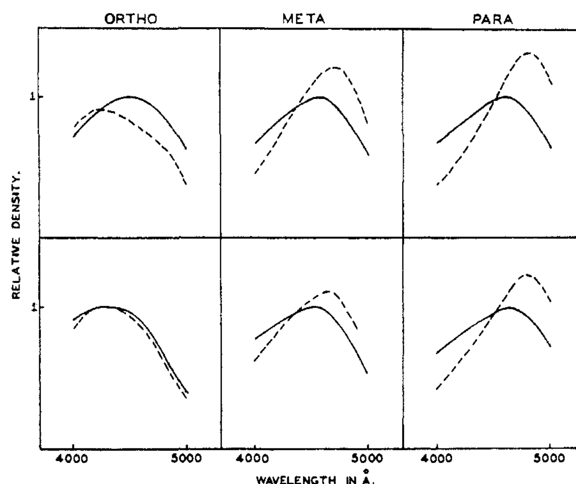
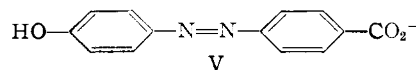


Fig. 3.—Top row: effects of human albumin on the spectra of ortho, meta and para methyl orange, (VI), (VII) and (I), respectively. Dye concentrations, 1.74 × 10^{-5}, 1.16 × 10^{-5} and 1.16 × 10^{-5} M, respectively. Bottom row: effects of human albumin on the spectra of ortho, meta, and para methyl red, (VIII), (IX) and (II), respectively; dye concentrations, 1.50 × 10^{-5}, 1.48 × 10^{-5} and 1.30 × 10^{-5} M, respectively; borate buffer, pH 9.2; protein concentration, 0.2%. American Cyanamid Co. albumin used in each case, except with (para) methyl orange, where lot 179-5x was used.

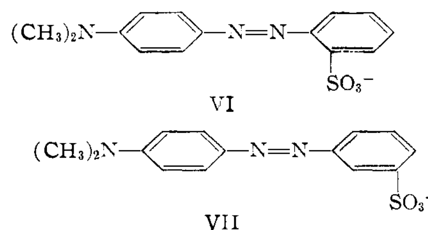
The effect of replacement of the dimethylamino substituent was examined by comparing the light absorptions of (II) and the 4′-hydroxyazobenzene-4-carboxylate ion (V). Bovine and human al-



bumin are not significantly different in their effects on the spectra at pH 9 of (V) and of the corresponding sulfonate ion. The interpretation is complicated in these cases, however, because the phenolic group is also ionized at pH 9 and hence the anions are doubly charged. Since the region of ionization overlaps the pH region of modification of human albumin, an unequivocal experiment could not be carried out.

Other variations in the dimethylamino substituent will be discussed more profitably in a later section.

Comparison of Isomeric Anions.—The isomeric position rather than the nature of the anionic substituent is important in determining the anomalous interaction with human albumin. The 4′-dimethylaminoazobenzene-2-sulfonate ion (VI), "ortho methyl orange," gives spectra (Fig. 3) with human albumin which are essentially identical with those of complexes with bovine albumin.



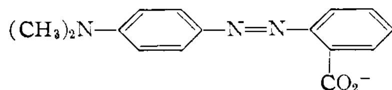
(18) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1945).

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

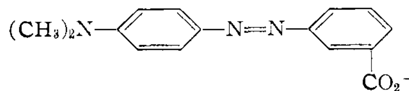
(20) I. M. Klotz, THIS JOURNAL, **68**, 2299 (1946).

On the other hand, "meta methyl orange" (VII) behaves like the para anion (Fig. 3).

This sensitivity to position is not limited to the sulfonate series. Thus methyl red (VIII) behaves like ortho methyl orange (Fig. 3), meta methyl red (IX) like meta methyl orange (Fig. 3). Corresponding studies with the phosphonic and arsonic

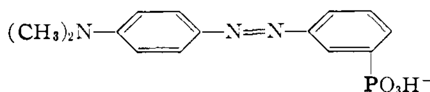


VIII



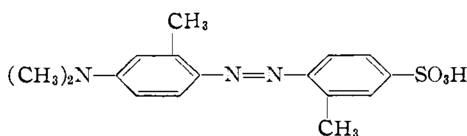
IX

acid analogs are incomplete. Nevertheless, the 4′-dimethylaminoazobenzene-3-phosphonate ion (X), behaves (Fig. 2) like meta methyl orange.



X

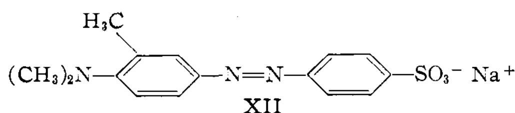
Interactions when Substituents Cause Steric Interference.—The exceptional behavior of the ortho compounds suggests blocking of the azo nitrogens by the ortho substituent, preventing interaction with the albumin molecule. To test this possibility, 4′-dimethylamino-2,2′-dimethylazobenzene-4-sulfonic acid (XI) was prepared, in which methyl groups block access to the azo nitrogens. Nevertheless, the spectrum with human



XI

albumin (Fig. 4) at pH 9 is essentially the same as that of methyl orange. Evidently the azo nitrogens are not directly involved in the interaction.

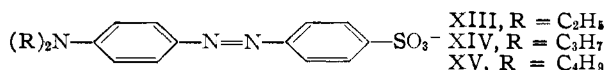
To test the importance of the dimethylamino group, o-toluidine orange (XII) was prepared, in which a methyl group blocks the amine nitrogen. This type of blocking, however, markedly alters the



XII

spectrum of the free anion, since it modifies the resonance between the dimethylamino substituent and the azobenzene skeleton, as has been shown by Remington[21] in simpler systems. Studies with this compound, therefore, were not pursued.

Alternatively, the spectra of ethyl (XIII), propyl (XIV) and butyl (XV) oranges, respectively, were examined (Fig. 4). A definite transi-



tion occurs in the effect of human albumin. With ethyl orange, human albumin at pH 9 still produces the exaltation and shift to higher wave length characteristic of its interaction with methyl orange (I). With propyl orange the exaltation is absent, but human albumin still does not lower the absorption to the extent that bovine albumin does. Finally with butyl orange the spectrum is essentially the same as with bovine albumin. Obviously as the size of the alkyl group is increased, access to the terminal nitrogen becomes more difficult until it is completely blocked with a group as bulky as butyl.

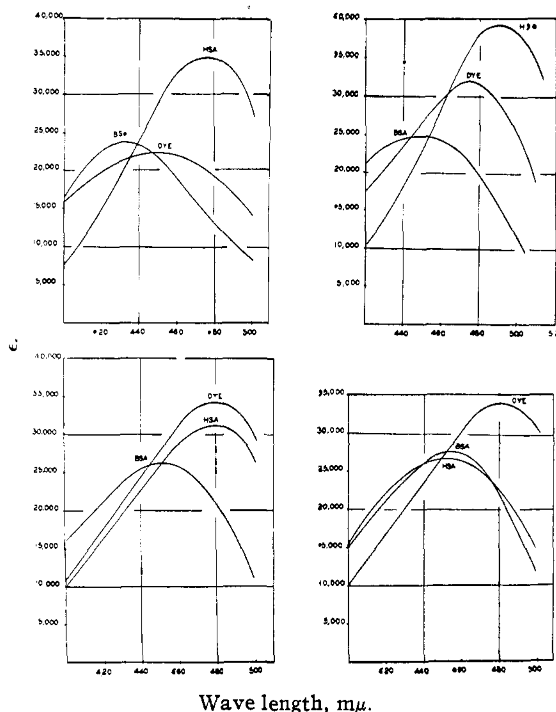


Fig. 4.—Spectra of 4′-dimethylamino-2,2′-dimethylazobenzene-4-sulfonate ion (XI) (upper left), ethyl orange (XIII) (upper right), propyl orange (XIV) (lower left), and butyl orange (XV) (lower right) and of their complexes with bovine serum albumin, BSA, and with human serum albumin (Cyanamid), HSA, at pH 9.2 in borate buffer; dye concentrations, 1.48×10^{-5}, 1.57×10^{-5}, 1.45×10^{-5} and 1.47×10^{-5} M, respectively; protein concentration, 0.2%.

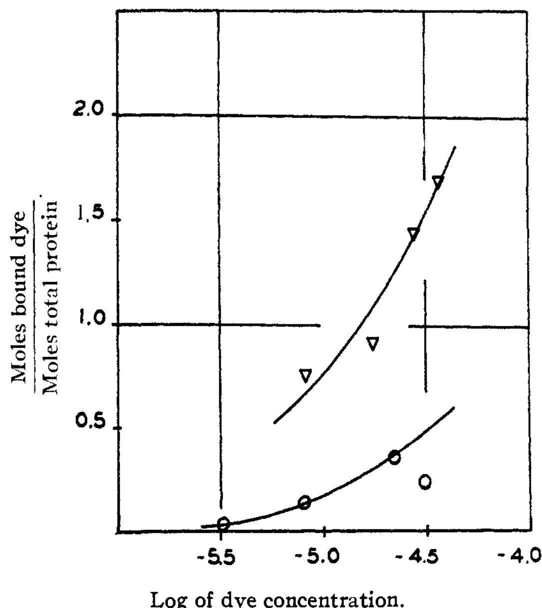
It should be emphasized that butyl orange still is bound by human (as well as by bovine) albumin, as is apparent from the shift in optical absorption. Thus blocking the terminal nitrogen atom does not eliminate all interaction with human albumin, for the electrostatic interaction with the sulfonate radical remains.

Quantitative binding data (Fig. 5) confirm the interpretation of the differences in spectra between methyl and butyl orange. Butyl orange is bound to a much smaller extent by human albumin at pH 9. The lesser affinity for the compound with the longer aliphatic side chain is particularly significant since it has been shown with simple carboxylic acids that affinity for albumin increases with chain length.[22] Thus it becomes apparent again that a specific interaction is possible when the

(21) W. R. Remington, THIS JOURNAL, **67**, 1838 (1945).

(22) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **167**, 407 (1947).

dimethylamino substituent is present, but not with the dibutylamino substituent in its stead. This interaction strengthens the affinity which exists normally due to the presence of the anionic substituent.

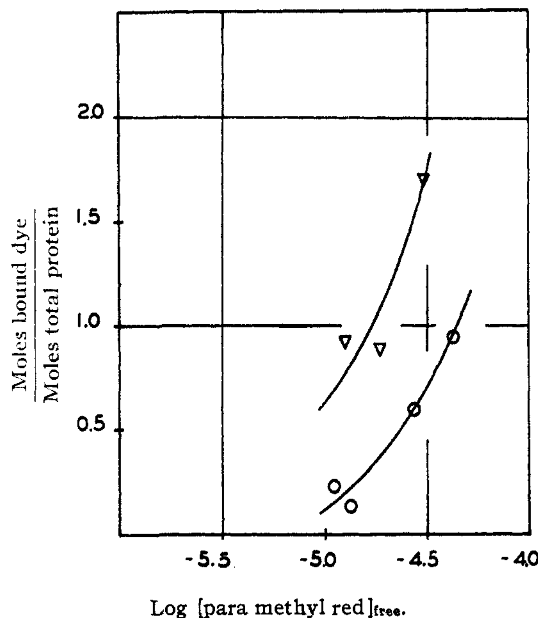


Log of dye concentration.

Fig. 5.—Comparison of affinity of human albumin (Cyanamid) for methyl orange, ▽, and for butyl orange, O; temperature 0°, borate buffer *p*H 9.2.

Protein Residues Involved in These Specific Interactions.—Among the dimethylaminoazobenzene anions described, those with a meta or para anionic substituent can be bound by human albumin at high *p*H at two points—at the anionic group and at the amino nitrogen. Since exclusion of the ortho compounds is not due to steric hindrance, it may be due to spatial orientation. The distance between amino nitrogen and anionic substituents differs markedly for the ortho as compared to the meta or the para isomer. With the ortho isomer the average distance (allowing for free rotation) is only 8.1 Å., whereas for the meta it is 11.5 A. and for the para, 12.8 A. It seems possible, therefore, that the two sites on human albumin capable of linking to the dimethylamino and anionic substituents, respectively, are approximately 12 Å. apart. The ortho isomer might not be capable of spanning the distance between sites while either the meta or the para could.

It seemed appropriate, therefore, to attempt to identify the binding sites involved. Insofar as the linkage of the $-SO_3^-$ ($-CO_2^-$, $-PO_3H^-$ or $-AsO_3H^-$) group is concerned, it was assumed that the same $\equiv NH^+$ sites are operative in human albumin as have been shown in bovine albumin.[23] Confirmation has been obtained with acetylated human albumin. The light absorption of the human albumin complex with para methyl red (II) drops markedly when the amino groups are (partially) selectively acetylated. Equilibrium dialysis also shows that binding is decreased (Fig. 6) on partial acetylation. The electrostatic effect of acetylation—increase in net negative charge—must also contribute to the decrease in binding of small anions, so that this comparison is not unequivocal proof of the participation of $\equiv NH^+$ sites.



Log [para methyl red]free.

Fig. 6.—Comparison of affinities for para methyl red (II) of human albumin (Cyanamid), ▽, and of acetylated human albumin, O; temperature 0°, borate buffer *p*H 9.2.

As for the linkage with the dimethylamino group, the character of the absorption band in the presence of human albumin[3] suggests that a hydrogen donor group from the protein (*e.g.*, from a hydroxy amino acid) participates. It has been suggested[3] that tyrosine groups might be especially effective, because resonance with the benzene ring favors a hydrogen-donating state for the phenolic hydroxyl. The spectrophotometric and binding effects of iodinated human albumin have therefore been investigated. In this protein the tyrosine groups are converted to iodotyrosine residues, in which the phenolic group, being more acidic, is ionized almost completely at *p*H 9 and hence has lost its hydrogen-donor character. Therefore. the anomalous spectrum of methyl orange with human albumin should disappear.

The spectra of methyl orange with "50%" iodinated and "100%" iodinated human albumin (Fig. 7) show that the anomalous exaltation drops with increasing conversion to tyrosine to iodotyrosine. The "100%" iodinated still does not behave like bovine albumin, but since 5 tyrosine residues are not iodinated, the observed curve is reasonable.

Comparison of the binding of methyl orange by these iodinated albumins and the parent albumin at *p*H 9 (Fig. 8) and 5.7 (Fig. 9) shows loss of affinity with increasing iodination, especially at *p*H 9. Some loss is to be expected because of increased electrostatic repulsion by iodoalbumin. This electrostatic factor, however, cannot be a major cause of decreased binding at *p*H 9 since the drop in affinity is at least of the order of magnitude ob-

(23) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

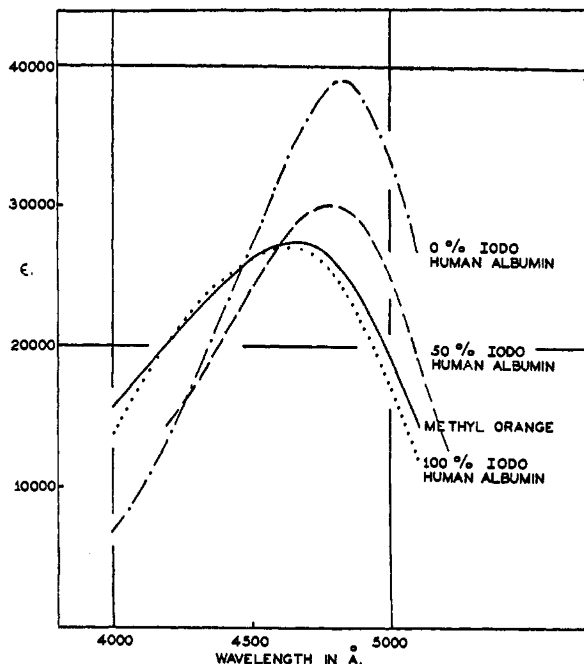


Fig. 7.—Spectra of methyl orange (I) and of its complexes with human albumin (lot Decanol 10) and with iodinated human albumins at *p*H 9.2 in borate buffer; concentration of methyl orange, 2.1×10^{-5} to 2.4×10^{-5} *M;* concentration of protein, 0.24%.

served on acetylation of human albumin, where the change in charge is far greater than that due to iodination. Thus iodination of albumin removes a site of specific interaction with methyl orange and with its meta and para analogs.

It seems reasonable to conclude, therefore, that the two sites involved in these specific interactions are tyrosine and cationic nitrogen side chains of human albumin. Furthermore, 12–13 Å. may be assigned as the distance between these types of residue. The anomalous spectra with the para and meta azo anions can be attributed to the fact that the distances between their substituents are between 11.5–13 Å., which allows these anions to span the distance between appropriate side chains. On the other hand, the ortho anions, containing substituents only 8 Å. apart, could not be linked by both types of side chain, in which case linkage occurs preferentially with a cationic residue, as indicated by the type of spectrum.

Cause of Configurational Change in Albumin.—As a mechanism by which human albumin makes available the additional sites detected at *p*H 9, the observation that the transition in effect of human albumin occurs near *p*H 7 suggests the ionization of residues, such as histidine, with *pK*'s in this region. Experiments with guanidinated albumin, however, indicate that such residues are not involved specifically.

Guanidination[16] of human albumin does not destroy its anomalous interaction with methyl orange. Spectra (Fig. 10) obtained with the guanidinated albumin are analogous to those of the parent protein, except that the *p*H region for the anomalous spectrum is shifted toward higher values (*cf.* Fig. 1 and 10). At *p*H's 7.6 and 9.2,

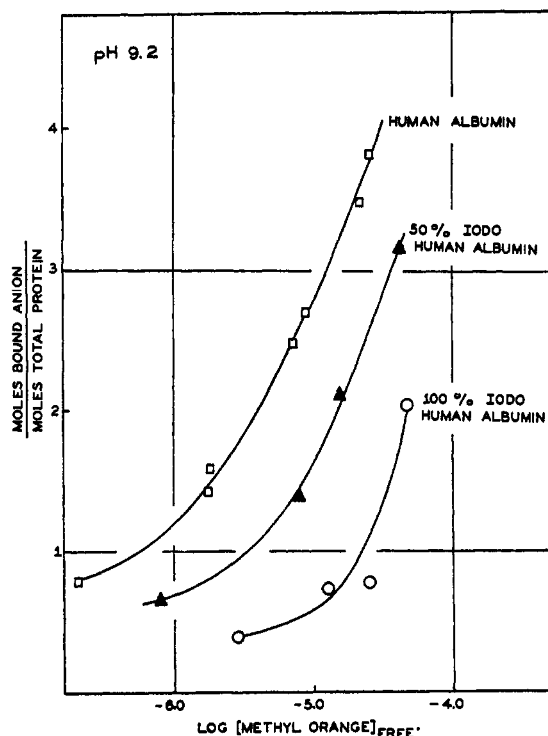


Fig. 8.—Comparison of affinities for methyl orange (I) of human albumin (lot 179-5x) and of iodinated human albumins (prepared from lot Decanol 10); temperature 0°, borate buffer *p*H 9.2.

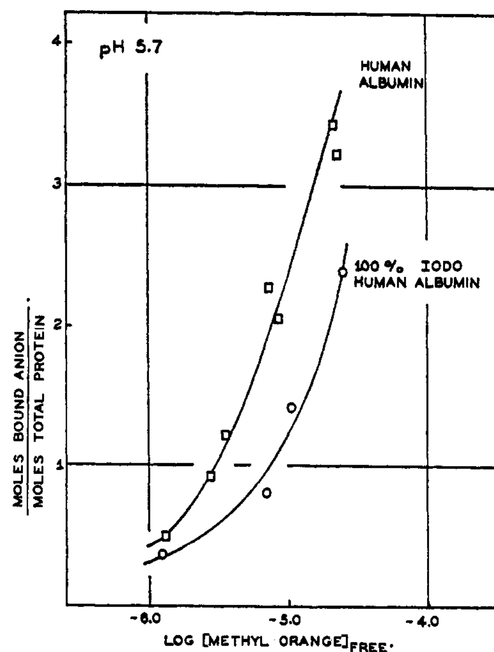


Fig. 9.—Comparison of affinities for methyl orange (I) of human albumin and of iodinated albumin at *p*H 5.7; temperature 0°, phosphate buffer.

respectively, the exaltation produced is less than that effected by the unmodified albumin. In 0.01 *M* NaOH the guanidinated albumin retains its full ability to produce the anomalous spectrum, whereas the unmodified protein loses almost all of its effect.

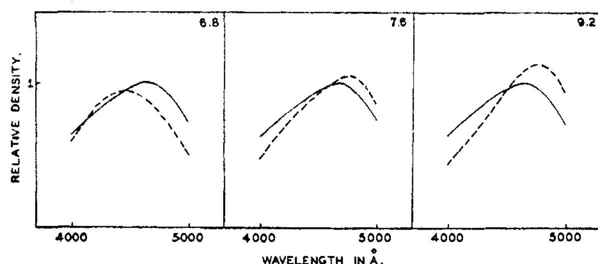


Fig. 10.—Effects of guanidinated human albumin on the spectra of methyl orange at a series of *p*H's (listed in corner of each figure); concentration of methyl orange, 1.15×10^{-5} *M*; concentration of protein, 0.16–0.20%.

Above *p*H 12, in 0.03 *M* NaOH, guanidinated albumin also loses essentially all of its effect.

If histidine groups were specifically involved, the transition region of the guanidinated protein should be shifted toward lower rather than higher *p*H's; for the guanidinated albumin carries a smaller negative charge than does the unmodified and hence should allow protons to be dissociated more readily.

The behavior of guanidinated albumin indicates, furthermore, that the transition in properties of human albumin is due to a configurational change caused largely by electrostatic factors. The effect of the modified protein appears shifted to higher *p*H regions primarily because the transition is spread over a wider *p*H interval. Both give a "normal" optical effect (Figs. 1 and 10) at *p*H 6.8. The anomalous interaction shows up at *p*H 7.6, guanidinated albumin producing a weaker effect. At *p*H 9 the unmodified protein reaches its maximum effect, whereas the guanidinated must attain much higher *p*H's. The course of ionization is parallel, the unmodified protein reaching a specified net negative charge at a lower *p*H. Furthermore, in the guanidinated, the rate of increase in negative charge is much less[24] in the *p*H region near 9 because of the scarcity of lysine groups.

It seems, therefore, that human albumin becomes either swollen or unfolded when the net charge reaches about −10. As the *p*H increases and the charge becomes more negative, electrostatic repulsion causes an even greater opening-up of the protein until a maximum is reached, as detected by the present criteria, at a total charge of about −30. The charge on guanidinated albumin, rising more slowly with increasing *p*H, reaches a value of −30 about 1 *p*H unit higher[24] than that required by the unmodified protein. In both cases, however, expansion or unfolding makes new sites available for interaction with anions. For certain anions some sites become available which are spaced at especially favorable distances and hence specific interactions come into play.

(24) C. Tanford, This Journal, **72**, 441 (1950).

Conclusions.—Several important aspects of ion-protein interactions are clarified by the present experiments. First, human albumin undergoes major configurational changes with changes in *p*H, making new sites available for interactions with ions of specific structural properties. Thus the effect of *p*H on binding affinity cannot be predicted simply from electrostatic changes.

Furthermore, these configurational changes are probably caused by electrostatic repulsions within the protein molecule when it attains a sufficiently high charge. If small ions possessing the same sign of charge as the protein are bound, they too would tend to increase the net charge on the large molecule, which again would swell or unfold and new binding sites would become available for further ion binding.

Although the specific interactions described here occur with human albumin, it seems likely that analogous ones also occur with bovine albumin. The specific anions studied fit the configuration of certain sites on human albumin. A suitable choice of anions with other structures should lead sooner or later to the discovery of analogous specificities in interactions with bovine albumin.

With spectrophotometric methods it is possible to assign these specific interactions to particular residues with more confidence than might be possible from studies of comparative binding affinities, making it feasible to establish distances between side chains through studies of interactions with ions of fairly rigid structure. A method thus seems available for mapping the topography of a native protein in solution.

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