TABLE III

SUMMARY OF THE PHYSICAL CONSTANTS OF CONALBUMIN AND IRON–CONALBUMIN

Protein	Molecular weight: Osmotic pressure	Molecular weight: Light scattering	Molecular weight: Sedimentation diffusion	s_{20w}, sec.$^{-1}$ × 10^{13}	D_{20w}, cm.^{2} sec.$^{-1}$ × 10^7	V, cm.^{3}	Specific refraction increment	f/f_0: a	f/f_0: b	f/f_0: c
Conalbumin	86,500	82,400	86,000	5.05	5.30	0.732	0.001964 (546 mμ) .001990 (436 mμ)	1.365	1.376	1.375
Iron–conalbumin	85,000	....	83,000	5.26	5.72	.732		1.310	1.277	1.295

understandable in light of the present work since they were dealing with the iron-free form, while Bain and Deutsch used the iron complex. In addition, their value would have been higher at a lower protein concentration. Actually, the value reported by Cann and Phelps fits very well on the s_{20w}–concentration curve for conalbumin reported herein (see Fig. 3).

The average molecular weight reported here for both forms of the protein by the three independent methods of measurement is 84,000 ± 2000. The agreement between the values obtained by the three different methods is strong evidence for the homogeneity of the preparations used and the reliability of the molecular weight reported. It is of interest that the extrapolated values of s_{20w}, the values of D_{20w}, and those of the frictional ratios all indicate the protein molecule to be of a more spherical shape when in the form of the iron complex; however, the greater dependence of the s_{20w} value on concentration in the case of the iron–conalbumin would indicate the reverse relationship to hold.

ST. PAUL, MINN.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

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

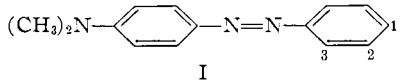
BY WARNER L. PETICOLAS[1] AND IRVING M. KLOTZ

RECEIVED JUNE 4, 1956

Previous observations of marked differences in the interaction of human albumin with isomeric molecules (I), containing an anionic substituent, –X, in positions 1, 2 and 3, respectively, have been ascribed to differences in the distances between $(CH_3)N$– and –X. The validity of this view has been bolstered by further studies with molecules of the type II in which chains of varying length and rigidity have been interposed between the ring and carboxyl group.

Introduction

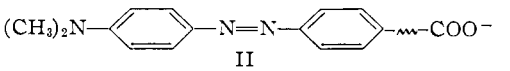
It was suggested recently[2] that it might be feasible to establish distances between side chains of proteins through spectrophotometric studies of interactions with ions of fairly rigid structure containing two substituents a known distance apart. Thus, it was shown that with human serum albumin, azo compounds of the structure I behaved



I

differently if an anionic substituent, –X, was in position 1 or 2 as contrasted to position 3. If –X is a $-COO^-$ group, for example, the spectrum of the complex of protein with the *meta* or the *para* dye (carboxyl in 2 or 1 position, respectively) showed a peak near 480 mμ, whereas that of the complex with the *ortho* compound had a maximum near 430 mμ. Evidence was presented that two side chains on the protein formed bonds with I when $-COO^-$ was at 1 or 2, but that only one of these was involved when the carboxyl was at 3. The critical feature seems to be the distance between the N of $(CH_3)_2N$– and the O of $-COO^-$, which is approximately 12 Å. for either the *m*- or *p*-carboxyl, but near 9 Å. for the *ortho*. The protein side chains involved in binding apparently are able to span a distance of 12 Å. but not one of 9 Å.

As a further test of this interpretation it seemed appropriate to separate the $(CH_3)_2N$– and $-COO^-$ by a spacing greater than 12 Å. to see if one could exceed the distance which the two protein side chains could span. A number of compounds have been prepared, therefore, in which chains of varying length and rigidity have been interposed between the ring and carboxyl group (structure II),



II

and the interactions of these dyes with serum albumin have been examined.

Experimental

Reagents.—4-(*p*-Dimethylaminophenylazo)-hydrocinnamic acid (VI) was prepared by the following sequence of reactions. Hydrocinnamic acid (Eastman Kodak Co. sample) was nitrated according to the procedure of Konek and Pacsu,[3] and the *p*-nitrocinnamic acid isolated was hydrogenated in a Parr bomb. The resultant *p*-aminohydrocinnamic acid was diazotized and coupled according to the general procedure of Fieser.[4] The dye thus produced (VI) was recrystallized several times from ethanol–water mixtures. It melted at 194–197° dec., and gave a neutralization equivalent of 306 (calcd. 297). *Anal.* Calcd. for $C_{17}H_{19}O_2N_3$: C, 68.66; N, 14.14. Found: C, 69.06; N,

(1) Pre-doctoral Fellow of the National Institutes of Health, 1951–1953.

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

(3) F. von Konek and E. Pacsu, *Ber.*, **51**, 855 (1918).

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

14.36. Chromatography of the dye on an activated alumina column revealed only one component.

4-(*p*-Dimethylaminophenylazo)-hippuric acid (IX) was obtained by diazotization of *p*-aminohippuric acid (Eastman Kodak Co. sample) followed by coupling with dimethylaniline according to the method of Fieser.[4] The ammonium salt of the dye was recrystallized several times from ammonium hydroxide and the acid was precipitated from a hot ammoniacal solution by the addition of dilute HCl. The dye was dissolved in a large volume of ethanol and the solvent was then allowed to evaporate to one-tenth of its initial volume. The crystals so obtained had a melting point of 232–233° and a neutralization equivalent of 318 (calcd. 316). *Anal.* Calcd. for $C_{17}H_{18}O_3O_4$: C, 62.56; H, 5.56; N, 17.17. Found: C, 62.49; H, 5.49; N, 17.20.

4-(*p*-Dimethylaminophenylazo)-cinnamic acid (VII) was obtained as follows. *p*-Nitrocinnamic acid (Eastman Kodak Co. sample) was reduced to *p*-aminocinnamic acid by ammoniacal ferrous sulfate according to the method of Gabriel,[5] and the amino compound so formed was diazotized and coupled with dimethylaniline. The dye, which was purified in the same way as IX, had a melting point of 252–253° and a neutralization equivalent of 299 (calcd. 295). *Anal.* Calcd. for $C_{17}H_{17}O_2N_3$: C, 69.13; H, 5.80; N, 14.23. Found: C, 68.75; H, 5.74; N, 14.07.

3-(*p*-Dimethylaminophenylazo)-cinnamic acid (VIII) was prepared from *m*-nitrocinnamic acid (Eastman Kodak Co. sample) by a procedure corresponding to that used for the *para* dye. The *meta* dye VIII had a melting point of 203–204° and a neutralization equivalent of 292 (calcd. 295). *Anal.* Calcd. for $C_{17}H_{17}O_2N_3$: C, 69.13; H, 5.80; N, 14.23. Found: C, 69.15; H, 5.87; N, 14.55.

Borate buffer, *p*H 9.2, was prepared from reagent grade $Na_2B_4O_7 \cdot 10H_2O$ to give an ionic strength of 0.15. Phosphate buffer, *p*H 6.9, was prepared from $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4 to give an ionic strength of 0.13.

The bovine serum albumin was a crystalline sample purchased from Armour and Co. Most of the experiments with human serum albumin were carried out with a sample obtained from Drs. P. H. Bell and R. O. Roblin, Jr., of the American Cyanamid Co. The spectra of the complexes with 4-(*p*-dimethylaminophenylazo)-cinnamic acid were also examined with a crystalline sample of human albumin (Decanol 10) obtained from Dr. W. L. Hughes, Jr., of Harvard University.

Determination of Acidity Constants.—The *pK*'s of the carboxyl group of the azo dyes were determined by glass-electrode titration with sodium hydroxide, in ethanol–water mixtures to increase the solubility of the dyes. The values so obtained (in ethanol–water) were then extrapolated to pure water solutions on the basis of the linear relation between *pK* and volume percentage alcohol found by Larson and Tomsicek[6] for the closely related azo dye *o*-methyl red. The values for pure water as solvent are

	$pK_{25°}$
p-(*p*-dimethylaminophenylazo)-hydrocinnamic acid	5.06
p-(*p*-dimethylaminophenylazo)-hippuric acid	4.20
m-(*p*-dimethylaminophenylazo)-cinnamic acid	4.70
p-(*p*-dimethylaminophenylazo)-cinnamic acid	4.80

From these *pK*'s it is evident that in buffers of *p*H 6.9 and higher each of the dyes is almost entirely in anionic form.

Absorption Spectra.—The absorption of light was measured with the Beckman spectrophotometer, model DU, at approximately 25°; 1-cm. cells were used. Extinction coefficients, ϵ, were calculated from the equation

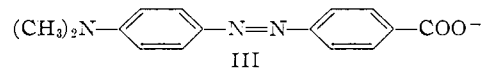
$$\epsilon = \frac{1}{cd} \log_{10}(I_0/I)$$

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. Spectra of the protein–dye complexes were generally taken at a dye concentration near 1.5×10^{-5} *M* and a protein concentration of about 2.5×10^{-5} *M*, each concentration being known accurately.

Dialysis Experiments.—The extent of binding was measured by dialysis technique described previously[7]; 10 ml. of 0.2% protein inside a cellulose-casing bag was equilibrated with 10 ml. of dye solution outside the bag. Each tube with a specific dye concentration was matched by a similar tube identical in all respects except that protein was omitted from the solution inside the bag. The non-protein tubes provided the information necessary to correct for the binding of dye by the bag. Equilibration in each tube was effected by mechanical shaking for a period of 24 hours.

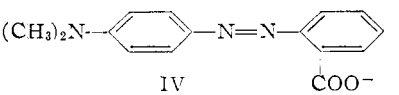
Results and Discussion

Spectra.—As has been shown previously,[2] if a carboxylate anion is attached to position 1 of structure I



III

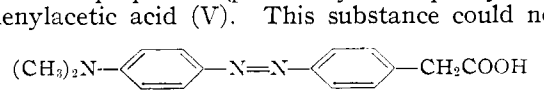
this behaves quite differently in complex with bovine serum albumin (BSA) than with human serum albumin (HSA). At *p*H 9, the absorption peak of the complex with the former protein is shifted to a wave length (435 mμ) shorter than that of the peak of the pure dye (465 mμ) in the same buffer, whereas with the latter protein, the peak is shifted to a higher wave length (480 mμ) and its intensity is increased substantially. For a variety of reasons,[2] including the observation that the high peak with human albumin is not obtained with the carboxylate ion in position 3 of structure I, there is firm



IV

basis for the interpretation that dye III is bound to the human albumin by linkages to both the $(CH_3)_2N$– and $-COO^-$ groups, whereas with dye IV a bond to the $(CH_3)_2N$– group is lacking. Evidently with the *ortho* dye IV the distance between substituents, about 9 Å., is too small to span the interval, about 12–13 Å., between the protein side chains involved in these specific linkages. One would expect, therefore, that if atoms were interposed between the carboxylate anion and the ring (as in II) so that the distance between $(CH_3)_2N$– and $-COO^-$ groups were increased sufficiently above 13 Å., an extension should be reached too great to span the approximately 13 Å. interval between the protein side chains involved.

As a first step in this direction, an attempt was made to prepare 4-(*p*-dimethylaminophenylazo)-phenylacetic acid (V). This substance could not



V

be crystallized and characterized, but the amorphous material in aqueous buffer at *p*H 9.2 showed the same type of spectrum with human albumin as does "*para* methyl red" (III). Evidently, the methylene group interposed in V does not increase the span between the extreme substituents sufficiently.

Two methylene groups were introduced, therefore, to produce 4-(*p*-dimethylaminophenylazo)-

(5) S. Gabriel, *Ber.*, **15**, 2291 (1882); S. Gabriel and M. Herzberg, *ibid.*, **16**, 2038 (1883).

(6) W. D. Larson and W. J. Tomsicek, THIS JOURNAL, **72**, 2774 (1950).

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

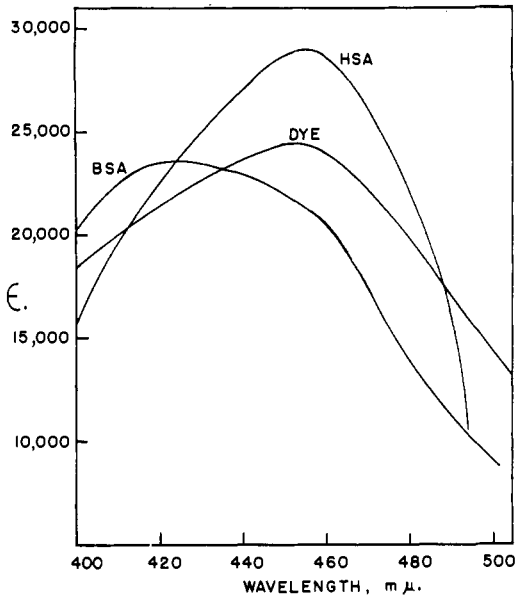


Fig. 1.—Absorption spectra of $(CH_3)_2N$—⟨⟩—N=N—⟨⟩CH_2CH_2COOH in buffer at *p*H 9.2 and 25°. Dye alone (dye); dye with bovine serum albumin (BSA); dye with human serum albumin (HSA).

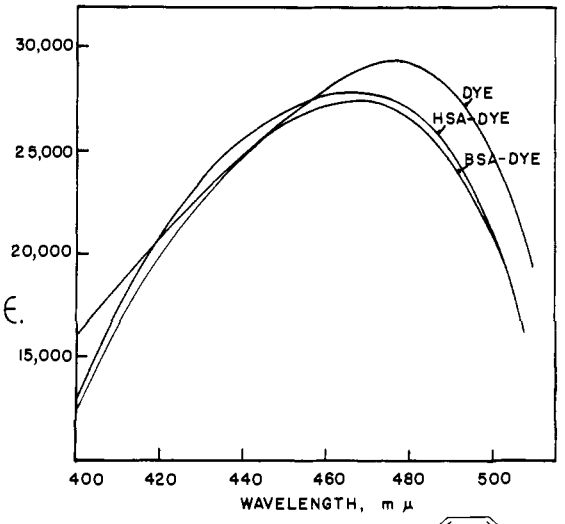


Fig. 2.—Absorption spectra of $(CH_3)_2N$—⟨⟩—N=N—⟨⟩—CH=CHCOOH in buffer at *p*H 9.2 and 25°.

dihydrocinnamic acid (VI). Contrary to initial

$(CH_3)_2N$—⟨⟩—N=N—⟨⟩—CH_2—CH_2—COOH

VI

expectation, this dye at *p*H 9.2 also showed the shift to the red with human albumin (Fig. 1) characteristic of dye III. After construction of molecular models of these dyes, however, it became apparent that compound VI, because of free rotation around the methylene groups, could readily curl around so that the span between $(CH_3)_2N$- and $-COO^-$ was reduced from a value of about 16 Å. at full extension to a distance of approximately 13 Å., that is, small enough to fit between the specific protein side chains involved.

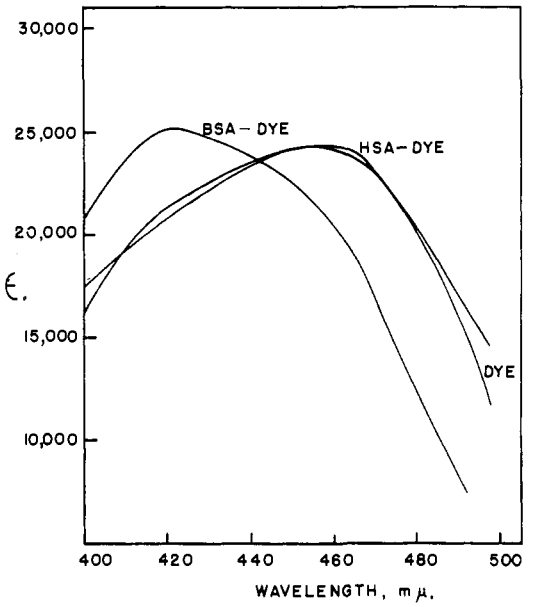


Fig. 3.—Absorption spectra of $(CH_3)_2N$—⟨⟩—N=N—⟨⟩(CH=CHCOOH) in buffer at *p*H 9.2 and 25°.

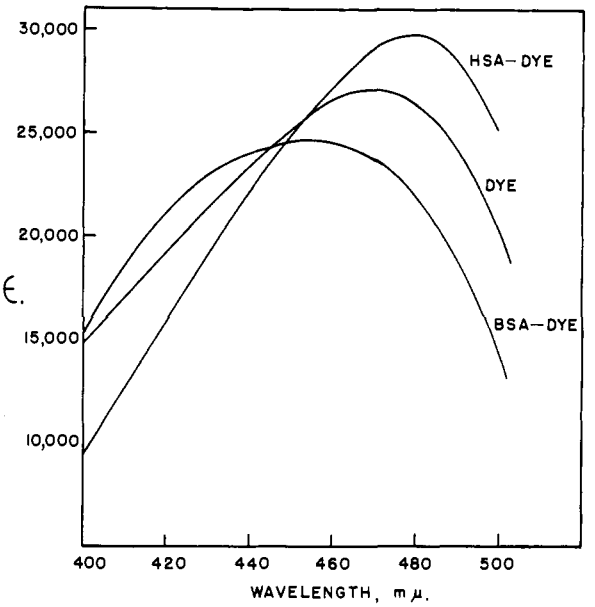


Fig. 4.—Absorption spectra of $(CH_3)_2N$—⟨⟩—N=N—⟨⟩—$CONHCH_2COOH$ in buffer at *p*H 9.2 and 25°.

One would expect, therefore, that if a *rigid* bridge could be interposed between the $-COO^-$ group and the ring so that no free rotation was possible, a fixed span[8] would be established. This objective was accomplished with the cinnamic acid analog of VI, 4-(*p*-dimethylaminophenylazo)-cinnamic acid (VII).

$(CH_3)_2N$—⟨⟩—N=N—⟨⟩(CH=CH—COOH)

VII

(8) Computations from models show that this span would be 15 Å.

TABLE I

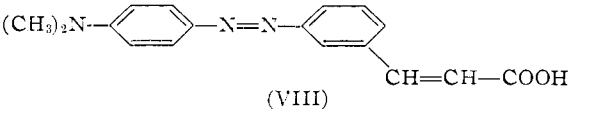
FREE ENERGIES, ENTHALPIES AND ENTROPIES FOR THE BINDING OF CERTAIN AZO COMPOUNDS BY SERUM ALBUMIN, BORATE BUFFER, *p*H 9.2

	Human serum albumin				Bovine serum albumin			
Cmpd.[a]	$\Delta F_1°$ at 0°, cal./mole	$\Delta F_1°$ at 25°, cal./mole	$\Delta H_1°$, cal./mole	$\Delta S_1°$, cal., mole^{-1} deg.$^{-1}$	$\Delta F_1°$ at 0°, cal./mole	$\Delta F_1°$ at 25°, cal./mole	$\Delta H_1°$, cal./mole	$\Delta S_1°$, cal., mole^{-1} deg.$^{-1}$
IV	−6770	−6940	−4900	7	−6740	−7310	− 500	23
III	−7150	−7450	−3900	12	−6860	−7500	+ 100	26
IX	−7290	−7320	−7000	1	−6290	−6530	−3700	10
VI	−7500	−7620	−6200	5	−6800	−7350	− 800	22
VII	−6850	−7340	−1500	20	−6880	−7350	−1800	19
VIII	−7480	....	....	..	−7540	....	....	..

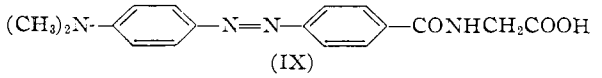
[a] See text for structural formulas.

Spectra of this dye (VII) with human albumin and bovine albumin (Fig. 2) are analogous to those with compound IV rather than III. With both proteins the optical shift is toward shorter wave lengths. Thus, it appears that with a long rigid molecule, VII, one can exceed the optimal span (near 13 Å.) between protein side chains responsible for the red shift, and the behavior observed becomes the same as that found with compound IV in which the substituents are too close together to attain the optimal span.

Spectra of two other dyes related to this series, 3-(*p*-dimethylaminophenylazo)-cinnamic acid (VIII) and 4-(*p*-dimethylaminophenylazo)-hippuric acid (IX), were also taken and fit the steric interpretation outlined. With VIII the



(VIII)



(IX)

distance between extremities has been shortened slightly, as compared with VII, yet the molecule is rigid. The observed spectrum (Fig. 3) with human albumin gives an indication of a red shift, as

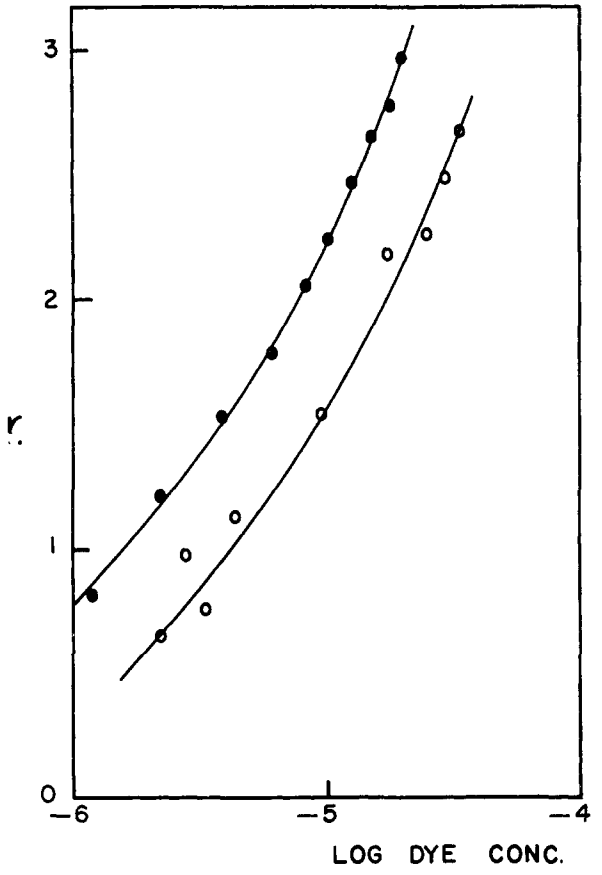


Fig. 5.—Binding of $(CH_3)_2N-C_6H_4-N=N-C_6H_4-CH_2CH_2COOH$ by human serum albumin at *p*H 9.2: ●, 0°; ○, 25°.

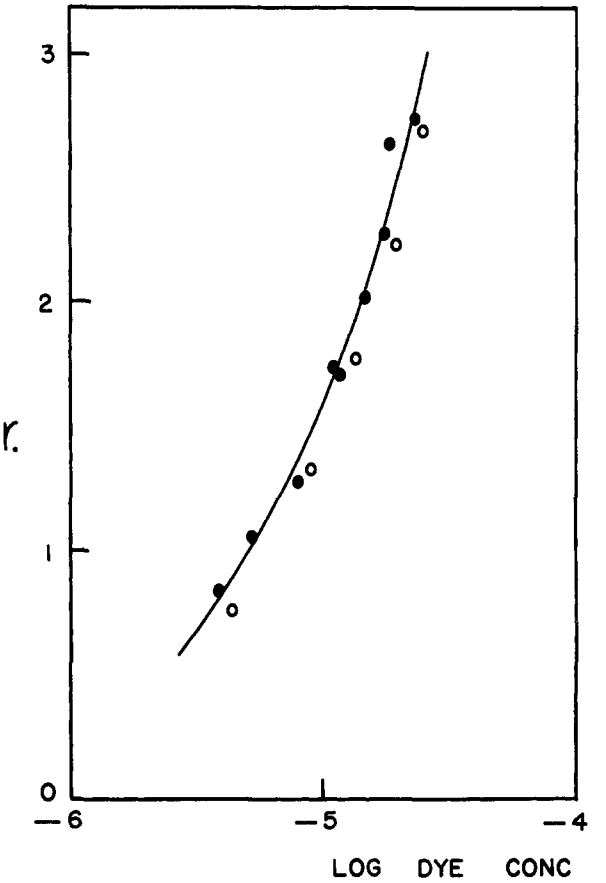


Fig. 6.—Binding of $(CH_3)_2N-C_6H_4-N=N-C_6H_4-CH_2CH_2COOH$ by bovine serum albumin at *p*H 9.2: ●, 0°; ○, 25°.

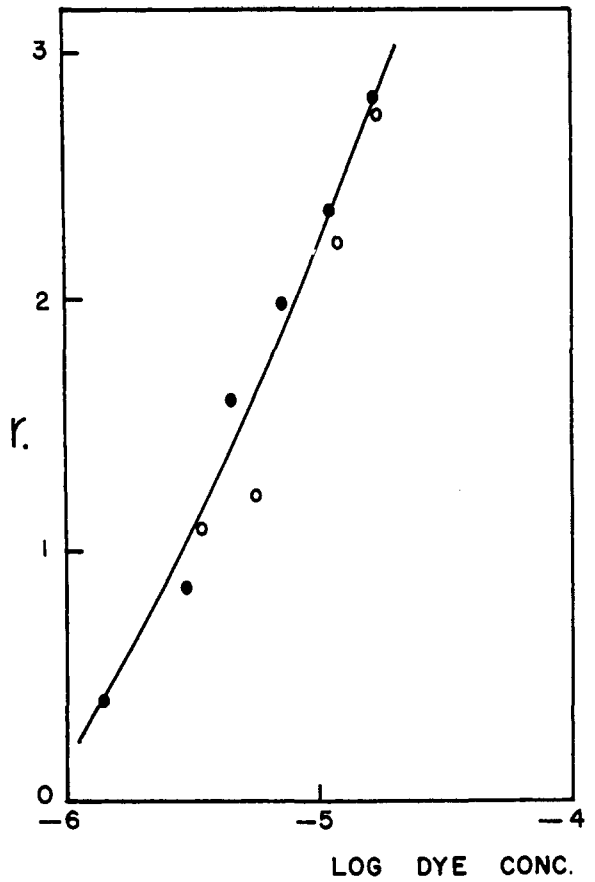


Fig. 7.—Binding of $(CH_3)N$—⟨⟩—N=N—⟨⟩—CH=CHCOOH by human serum albumin at *p*H 9.2: ●, 0°, ○, 25°.

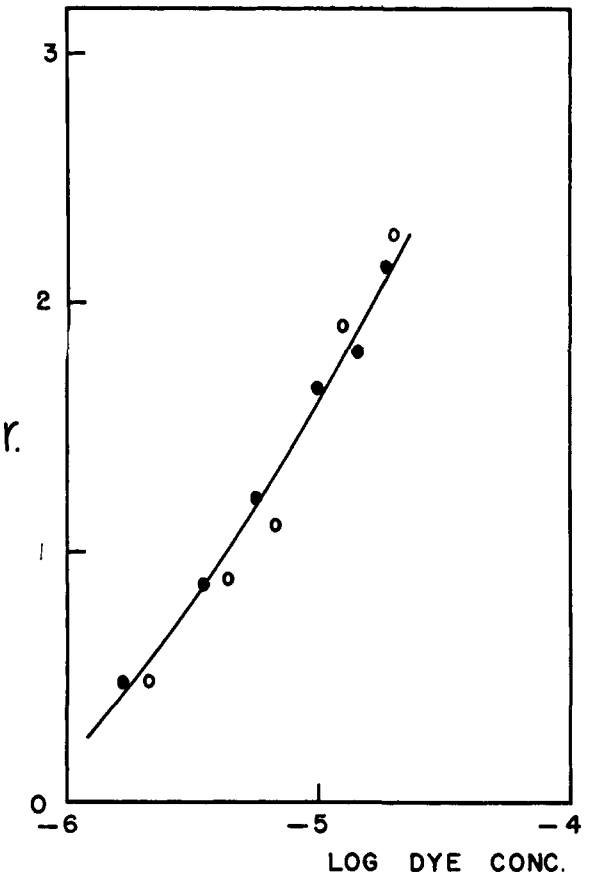


Fig. 8.—Binding of $(CH_3)_2N$—⟨⟩—N=N—⟨⟩—CH=CHCOOH by bovine serum albumin at *p*H 9.2: ●, 0°; ○, 25°.

compared to the effect of bovine albumin, but VIII is not short enough to really fit well and produce a spectrum like that of the complex with III. On the other hand, compound IX, possessing the attribute of free rotation in the right-hand side chain, is definitely able to curl around and reduce its span to about 13 Å. Thus, it shows an absorption spectrum with human albumin (Fig. 4) very similar to that of III or VI.

Comparison of Binding Energies.—The extents of binding of two of the dyes investigated are represented in Figs. 5–8 in terms of the average number of bound ions per mole of protein, *r*, *versus* the logarithm of the concentration of free anion, A. For all of the dyes, free energies of binding were evaluated from such data[9] by methods described in detail previously.[2,7] If the binding sites all have the same intrinsic constant, *k*, then

$$\lim_{A \to 0} \left[\frac{r}{(A)}\right] = nk \qquad (1)$$

where *n* is the total number of available sites for the anion on one protein molecule. As this equation implies, one may plot $r/(A)$ *versus* (A) and obtain *nk* from the extrapolated intercept at (A) = 0.

(9) For complete binding data see W. L. Peticolas, Ph.D. Dissertation, Northwestern University, 1954; *Dissertion Abstracts*, **14**, 2213 (1954).

For a single set of binding sites, furthermore

$$nk = k_1 \qquad (2)$$

where k_1 represents the equilibrium constant for the first anion complexed with the protein. Thus k_1 may be calculated from an extrapolation of the experimental binding data and hence $\Delta F_1°$ may be computed from the general thermodynamic relation

$$\Delta F_1° = -RT \ln k_1 \qquad (3)$$

Values of $\Delta F_1°$ are summarized in Table I. Entropy changes computed from the relation

$$\Delta S_1° = -(\partial \Delta F_1°/\partial T)_P \qquad (4)$$

and enthalpy changes obtained from

$$\Delta H_1° = \Delta F_1° + T\Delta S_1°$$

are also listed in Table I. It has been assumed in these calculations that $\Delta H°$ and $\Delta S°$ are temperature-independent over the range of 0–25°.

Examination of the free energies of binding with human albumin at 0° shows a correlation with the spectroscopic behavior of the azo compound. Compounds IV and VII, which are too small and too large, respectively, to give the anomalous peak with human albumin at *p*H 9.2 also show a lower free energy of binding. This correlation is probably fortuitous, however, since it is no longer clear-cut in the data for 25°. Likewise, there is no

parallelism between the enthalpy, or entropy, of binding and the type of spectrum given by the complex.

It is of interest, nevertheless, to note that compounds IX and VI, with the flexible side chains, show the smallest entropies of binding. Since the spectroscopic behavior of IX and VI indicates that the flexible side chains are twisted backward in order to accommodate themselves to the specific protein sites involved in the binding, this restriction on free rotation may reduce the configurational entropy and thus compensate in large part for the increase in entropy due to release of water molecules on binding. In line with this view are the substantially larger entropies of binding of these compounds, IX and VI, by bovine albumin. With the bovine protein, the spectra are "normal" and show no isomeric specificities, so that there is not as restricted a configuration for the flexible side chains of these bound molecules.

Conclusions.—The thermodynamic quantities for the binding process thus reflect the contributions of all portions of the small molecule in the formation of the complex with the protein. If the substituents on a particular molecule are not situated in positions sterically favorable for interaction with particular side chains of the protein, binding may still be strong because of interaction with other portions of the macromolecule.

Spectra, on the other hand, reflect the interactions of particular sites on the protein with specific substituents of the bound molecule, even though these bonds may not be the major contribution to the energetics of binding. Since these are specific interactions, the spectra are very sensitive to steric effects. With the present series of molecules two types of interaction are observed. Molecules (I, 3) and (VII) form one type of spectroscopic complex, whereas (I, 1) and (I, 2) comprise a second type. The distances between substituents of each molecule of the latter pair are essentially the same, 12–13 Å.; the separations of substituents in the former pair are markedly different, 9 and 15 Å., respectively, and yet they behave alike. Spectra of the complexes thus reflect very clearly differences in distances between substituents of the small molecule and hence between specific sites on the protein.

Acknowledgments.—These investigations were assisted by grants from the Office of Naval Research, Department of the Navy (NR 124-054), and from the Rockefeller Foundation.

EVANSTON, ILLINOIS

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES]

A Study of Compound Formation between BF_3 and Proteins[1]

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A detailed study of the nature of the complexes formed by solid proteins on sorbing BF_3 has been carried out. The first method that was used was to find the amounts of BF_3 bound irreversibly by several proteins and see if any correlation existed between these values and the amino acid compositions of the proteins. The method proved inconclusive since the BF_3 irreversibly bound was a function of the temperature of desorption in all the cases studied. The more rigorous method of examining the desorption isotherm for isobaric regions was also undertaken. Owing to the practical difficulties in this method only the egg albumin–BF_3 system was studied. No stoichiometric compounds were observed. From the continuity in the desorption rate data, and the similarity between such data for all the proteins, it was concluded that stoichiometric compounds did not exist in any of these systems. A hypothesis as to the nature of protein–BF_3 complexes is proposed. Supporting evidence is obtained from the fit of the data to the Freundlich equation, and the interaction of poly-γ-benzyl-L-glutamate and Nylon with BF_3.

Among the polar gases whose interaction with proteins is of interest from the point of view of compound formation, BF_3 offers much scope for investigation. It is a strong Lewis acid, which can form coördinate links with the oxygen, nitrogen, and possibly the sulfur atoms in proteins.[2] Sufficient information is available in the chemical literature to indicate that in simple organic compounds the same functional groups which are found in the amino acid residues constituting the protein chain, do form stoichiometric compounds with BF_3. Thus the ω-amino group in lysine can be expected to react with 1 mole of BF_3 in the manner of ethylamine.[3]

Though the guanidino group in arginine, and the imidazole group in histidine contain 2 nitrogen atoms apiece, it is probable from steric considerations that they would interact with only one mole of BF_3 each. The work of Landolph[4] and Bowlus and Nieuwland[5] indicates that amides and BF_3 form 1:1 compounds. Hence the amide groups in proteins can be expected to bind BF_3.[6]

Both oxygen and sulfur atoms have been known to form coördination compounds with BF_3. Hence the oxygen atoms in the alcohol (serine, threonine), phenol (tyrosine), and carboxyl (aspartic and glutamic acids; also the ester group in poly-γ-benzyl-L-glutamate) groupings of a protein are all poten-

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(2) Carbon acts as a donor only in olefinic compounds. These are not encountered in proteins.

(3) C. A. Kraus and E. H. Brown, THIS JOURNAL, **51**, 2690 (1929).

(4) F. Landolph, *Ber.*, **12**, 1578 (1879).

(5) H. Bowlus and J. A. Nieuwland, THIS JOURNAL, **53**, 3835 (1931).

(6) Tryptophan, which contains an indole group, has not been considered, since, among the proteins that were studied, it amounts to only 0.16 mmole/g. in fibrin and less than 0.1 mmole/g. in all the other cases.