ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF PEDIATRICS, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA AND THE CHILDREN'S HOSPITAL OF PHILADELPHIA]

Structural Effects of Anionic Azo Dyes on Serum Albumin[1]

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RECEIVED AUGUST 12, 1957

The effect of a number of anionic azo dyes on the optical rotation of human serum albumin was determined at *p*H 7.4 and room temperature. The effect of dyes having the general structure *p*-benzeneazobenzoylaminoacetic acid depends on the substituent occupying the *para*-position of the terminal phenyl group. A H or a OH group in this position increases the levorotation, the latter to −90°, whereas a dimethylamino group lowers it in one case to −16°. In the case where both the D- and the L-isomers of the same dye were tried the rotatory effect was different for the two isomers. The effect also depends on the protein employed since the rotatory patterns obtained with bovine serum albumin are markedly different from the changes observed with human serum albumin. The effect of the dimethylamino group in lowering the levorotation is also noted in the group of dyes related to methyl orange. If the terminal substituent is a OH group with the anionic group on the opposite end, the levorotation is increased when the anion is carboxylic or sulfonic acid, but not when it is arsonic acid. When the anionic group is in the *ortho* rather than in the *para*-position to the azo group the effect is generally smaller. The rotatory effects are completely reversed upon removal of the dye. Rotatory dispersion measured in two cases shows normal dispersion for a dye causing a large levorotation and highly anomalous dispersion for a dye which strongly decreases the levorotation. The large rotatory changes are accompanied by very small changes in reduced viscosity. Measurement of dye binding at a single total dye concentration shows the tendency for strongly bound dyes to cause the largest deviations from the native rotation. These results are interpreted as structural changes in the albumin molecule caused by the interaction with dye molecules, involving the stabilization of configurations varying in helical content. The variety of rotatory patterns observed provide experimental evidence for the flexibility of the albumin molecule and, less directly, for the concept of configurational adaptability.

The interaction of small molecules with proteins has received much attention in recent years, partly because of its importance for the mechanism of many biological processes. Most of this work was directed toward the characterization of the interaction itself, *e.g.*, the number and identity of the combining sites and the forces involved in the interaction. Except for the case of detergent–protein interaction[2] no direct experimental attempt was made to establish structural changes in the interacting protein molecule, although such changes were suggested by several authors on theoretical grounds. The basis for this assumption was furnished by a number of observations, among which the most important were the following: (1) serum albumins do not show a plateau in the amount of dye bound with increasing free dye.[3] To account for this observation it was proposed that the interaction causes the molecule to "open up" or unfold, thus permitting access to new binding sites. (2) The large entropy change that regularly accompanies the association of anionic dyes with serum albumin and which makes up most of the free energy of association has been attributed by Karush[4] to the rupture of intramolecular bonds and thus to a change in the organization of the protein. (3) The recent work on the interaction of two optically isomeric azo dyes (referred to as I^{D}_{d} and I^{d}_{L} in the present paper) with bovine and human serum albumin[4,5] demonstrated the capacity of the combining regions of albumin to assume configurations complementary to the contours of the isomeric dye molecules. This configurational adaptability involves changes in the relative positions of the side chains and thus reflects subtle structural changes in the organization of the molecule.

The present work was undertaken with the intention of obtaining experimental evidence of a more direct nature concerning the structural changes that accompany interaction with anionic azo dyes. For this purpose the measurement of optical rotation was chosen, since optical activity is sensitive to relatively fine structural changes and has proven of value in previous work dealing with the interaction of anionic detergents with serum albumin.[6] In two cases rotatory dispersion was also measured in order to provide more detailed structural information. Viscosity measurements also were carried out to detect gross changes in the shape of the protein. In order to obtain a relative measure of dye–albumin affinity, binding experiments were done at a single total dye concentration. Finally, shifts in the wave length of maximum light absorption by the dyes in the presence of albumin were correlated with the rotatory changes.

For purposes of comparison, the dyes used were divided into four groups on the basis of structural similarities and the kind of structural variation involved. Group I consists of dyes possessing the *p*-benzeneazobenzoylaminoacetic acid group. The variables here are the *para*-substituents on the terminal phenyl group. Members of group II have the dimethylamino group in the *para*-position of the terminal phenyl group, the variable being the nature and the position of the charged group. Group III is designed to demonstrate the effect of the number and position of the hydroxyl group on dyes having different acidic groups. Group IV contains miscellaneous dyes.

Experimental

Human serum albumin (HSA) was contributed by Cutter Laboratories and was 99% pure by electrophoretic analysis. Crystallized bovine serum albumin (BSA) was the product of Armour Laboratories (Lot No. P67908). An approxi-

(1) These studies were aided by a grant from The National Science Foundation and by a research grant (H-869) from The National Heart Institute of The National Institutes of Health, Public Health Service.

(2) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 195 (1945).

(3) I. M. Klotz in "The Proteins," Vol. IB, Eds. H. Neurath and K. Bailey, Academic Press, New York, N. Y., 1953, p. 773.

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

(5) F. Karush, THIS JOURNAL, **76**, 5536 (1954).

(6) G. Markus and F. Karush, *ibid.*, **79**, 3264 (1957).

mately 9% stock solution of the protein was prepared in distilled water, adjusted to pH 7.4 with NaOH and stored in the frozen state. Its concentration was determined by micro-Kjeldahl analysis. The experiments with BSA were done in 0.05 M phosphate buffer, pH 7.0, those with HSA in a mixture of 0.15 M NaCl and 0.02 M phosphate buffer, pH 7.4.

p(p-Dimethylaminobenzeneazo)-benzoylaminoacetic Acid (I_a).—The preparation of this dye was described by Karush.[4]

DL-Phenyl-(p-(benzeneazo)-benzoylamino)-acetic acid (I_b) was prepared by coupling DL-α-aminophenylacetic acid and p-phenylazobenzoyl chloride at pH 11. It was recrystallized from 50% ethanol; m.p. 206° with decomposition.

DL-Phenyl-(p-(p-hydroxybenzeneazo)-benzoylamino)-acetic acid (I_c) was prepared by diazotization of DL-phenyl-(p-aminobenzoylamino)-acetic acid and coupling with phenol at pH 11; m.p. 196–198° with decomposition.

D- and L-Phenyl-(p-(p-dimethylaminobenzeneazo)-benzoylamino)-acetic Acid (I_d^D) and (I_d^L).—The preparation of these dyes was described by Karush.[4]

Sodium p-(p-dimethylaminobenzeneazo)-benzenesulfonate (II_a), Eastman, was twice recrystallized from water.

Sodium p-dimethylaminoazobenzene-p'-carboxylate (II_b), Eastman, was twice reprecipitated as its acid.

Sodium p-dimethylaminoazobenzene-o'-carboxylate (II_c), Eastman, was twice recrystallized from 95% ethanol in the acid form.

p-Phenylazobenzoic acid (III_a) was prepared by treating p-phenylazobenzoyl chloride with 10% NaOH, m.p. 247°.

p-(p-Hydroxybenzeneazo)-benzoic acid (III_b) was prepared by coupling of the diazotized p-aminobenzoic acid with phenol; m.p. 260–261° with decomposition.

o-(p-Hydroxybenzeneazo)-benzoic acid (III_c) was prepared as the above; m.p. 206° with decomposition.

Sodium p-hydroxyazobenzene-p'-sulfonate (III_d), Eastman, was twice recrystallized from 95% ethanol.

Sodium 2,4-dihydroxyazobenzene-4'-sulfonate (III_e), Eastman, was recrystallized from absolute ethanol.

p-(p-Hydroxybenzeneazo)-phenylarsonic acid (III_f) was prepared by coupling diazotized p-arsanilic acid with phenol at pH 11. It was three times reprecipitated; m.p. > 300°.

p-(2-Hydroxy-5-methylphenylazo)-benzoic Acid (IV_a).—The preparation of this dye was described by Karush.[7]

o-(2-Hydroxy-5-methylphenylazo)-benzoic acid (IV_b) was a gift from Dr. Malcolm Siegel.

p-(1-Azo-2-naphthol)-benzoic acid (IV_c) was prepared by the coupling of diazotized p-aminobenzoic acid to β-naphthol. It was recrystallized from ethyleneglycol monomethyl-ether.

The dyes were made up in 5×10^{-3} M stock solutions. Stock solutions of the carboxylic acid dyes contained 1×10^{-2} M NaOH.

Optical rotations were measured at room temperature with a Bellingham and Stanley polarimeter which could be read to 0.01 of a degree, using a sodium vapor lamp and a 100 mm. cell. The specific rotations for colorless solutions are accurate to ±1°. In the presence of higher dye concentrations the error could be as much as ±2°. Solutions for the measurement of optical rotations were made up in 5 or 10 ml. volumes and were 0.9% with respect to protein.

Rotatory dispersion was measured on a Rudolph dispersion polarimeter. The use of this instrument was kindly arranged by Dr. E. Mihályi of The National Institutes of Health, Bethesda, Md.

Viscosities were measured in a capillary viscometer, made according to Schachman,[8] in a 25° water-bath. The flow time for water was 140 sec. Blank solutions differed from the samples only by the absence of protein.

Dye binding was measured by the method of equilibrium dialysis. Ten ml. of a 0.92% HSA solution containing 0.15 M NaCl and 0.02 M phosphate buffer, pH 7.4 were dialyzed against 10 ml. of the same salt–buffer mixture containing the dye at 1×10^{-3} M concentration. Equilibration was carried out on a rocking device in a 25° water-bath for 24 hr. All measurements were done on duplicate samples. In the calculation of the bound dye the molecular weight of albumin was taken as 65,000. The equilibrium concentration of the free dye was determined by spectrophotometric analysis of the 5-fold diluted outside solutions on the basis of previously determined molar extinction coefficients. The shift in the wave length of the absorption maximum of the bound dyes was determined on the bag contents without dilution by reducing the path length with a quartz spacer. The values of r (moles of dye bound per mole of albumin) were not corrected for the adsorption of the dye to the cellulose casing since it was estimated that the error would not exceed 3%.

(7) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(8) H. K. Schachman in "Methods of Enzymology," **4**, Eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York, N. Y., in press.

Results and Discussion

Group I.—Figure 1 summarizes the optical rotations, as a function of the total dye concentration, obtained with this group of dyes. The most striking difference exhibited here is shown by the OH and the $(CH_3)_2N$ substituted dyes, the former raising the levorotation to 90°, the latter lowering it to 19° in one case. Dye I_b with only a H atom at the corresponding site occupies a position intermediate between these two groups. The similarity of the patterns obtained with I_a and I_d^D is noteworthy because it indicates that the rotatory effect is only slightly influenced by the presence of the phenyl group on the charged end of the molecule and emphasizes the importance of the terminal substituent in determining the sign of the rotatory change.

Figure 2 shows the results obtained with D- and L-forms of dye I_d in solutions of bovine and human serum albumin at pH 7. The species difference exhibited in the general character of the rotatory curves probably is related to differences in binding of these dyes by the two albumins as shown by Karush.[4,5] The initial rise in levorotation terminating in the sharp peak in the BSA curves is absent in HSA, reflecting differences in the fine structure of the two proteins. The extent of the structural change caused by the binding of the D-isomer is greater in both proteins as judged by the greater deviations from the native $[\alpha]_D$ of −64°. The sharp peak in the I_d^L–BSA curve occurs at $r = 3.6$, *i.e.*, in the region of the inflection of the binding curve in Fig. I of ref. 4, thus strengthening the inference that a structural transformation takes place in the BSA molecule in this region of the binding curve. Similar correspondence for the D-dye at $r = 5.8$ is absent, but it should be noted that such a transformation for the binding of the D-dye, though obscured on the binding curve by the large negative slope, was considered to occur.[5]

Group II (**Fig. 3**).—The three dyes placed in this group possess the dimethylamino end. The effect of this substituent, as it was in the previous group for HSA, is an initial lowering of the levorotation. The structural similarity of methyl orange and p-methyl red is reflected in their rotatory patterns. The sulfonate group, however, appears to be slightly more effective in bringing about the analogous rotatory change. The smaller effect obtained when the carboxylate group is on the *ortho*-position to the azo linkage occurs also in the next groups and is accompanied by decreased binding relative to the *para*-analog. This behavior, first described by Klotz, Burkhard and Urquhart[9] was explained by them in terms of the distances between the dimethylamino and the carboxylate groups of the molecules, 12 Å. when the carboxy-

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

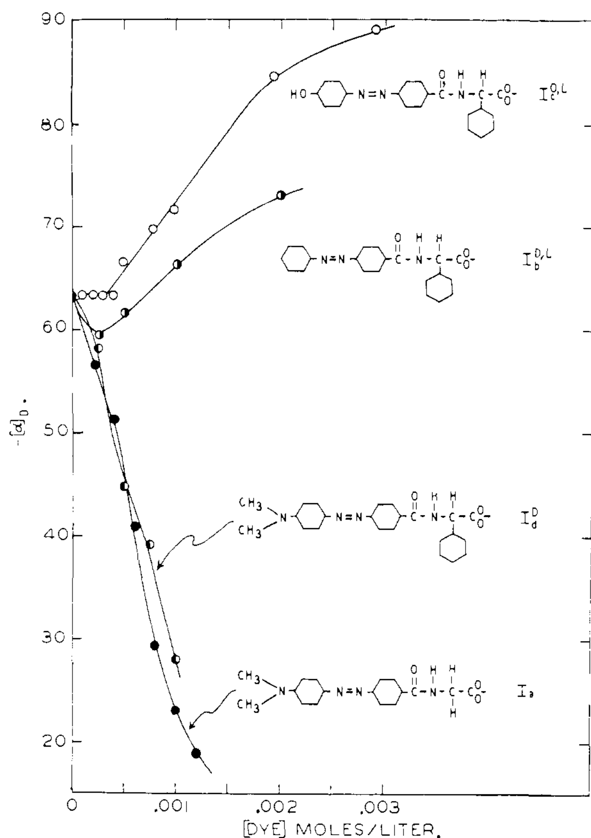

Fig. 1.—Specific rotation of 0.95% HSA as a function of the concentration of group I dyes in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4.

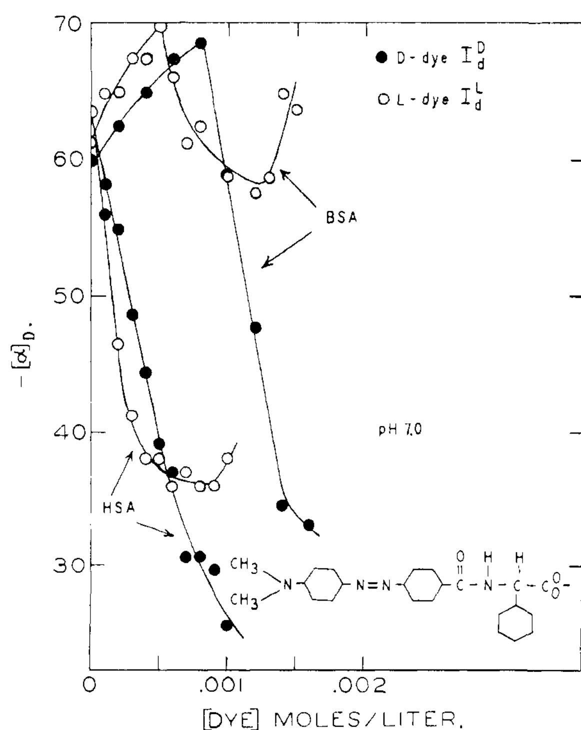

Fig. 2.—Specific rotation of 0.95% HSA and 0.82% BSA as a function of the concentration of I_d^D and I_d^L dyes in 0.05 M phosphate buffer, pH 7.0.

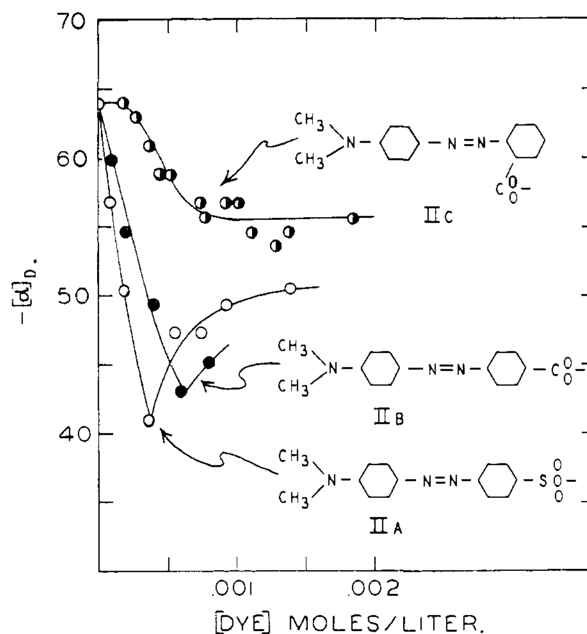

Fig. 3.—Specific rotation of 0.95% HSA as a function of the concentration of group II dyes in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4.

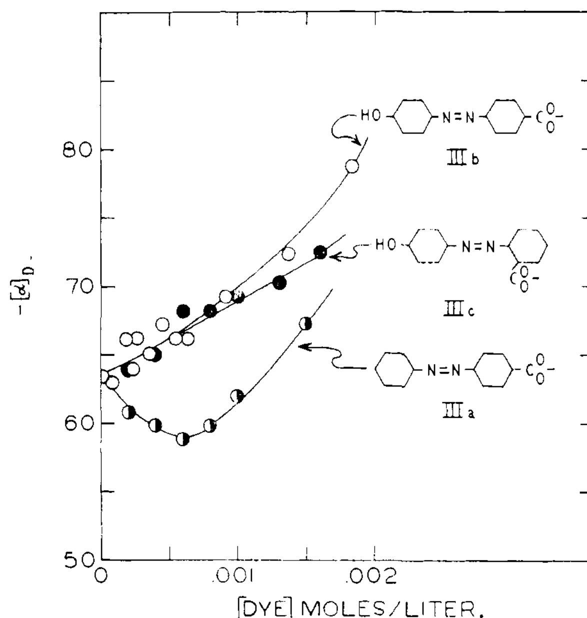

Fig. 4.—Specific rotation of 0.95% HSA as a function of the concentration of Group III dyes in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4.

late is in the *para*-position with respect to the azo group, but only 10 Å. when the carboxylate is *ortho* to the azo linkage.

Group III.—The superiority of the OH over the H substituent in increasing the levorotation, found in the case of dyes I_c and I_b, is again demonstrated for III_b and III_a in Fig. 4. It is surprising that III_c with the carboxylate in the *ortho*-position, though poorly bound, appears to be very effective in raising the levorotation. The sulfonic acid dye III_d (Fig. 5) is slightly more effective than its carboxylic acid analog, a behavior paralleled by

the methyl orange–*p*-methyl red pair. The corresponding arsonic acid derivative III_f is both weakly bound and ineffective in changing the rotation. The weak binding of the arsonic acid analog of methyl orange was also noted by Klotz, *et al.*[9] The poor binding and lack of substantial rotatory effect of the sulfonic acid dye III_e differing from III_d only by the additional OH group in the *ortho*-position may be due to the greater water solubility of this compound.

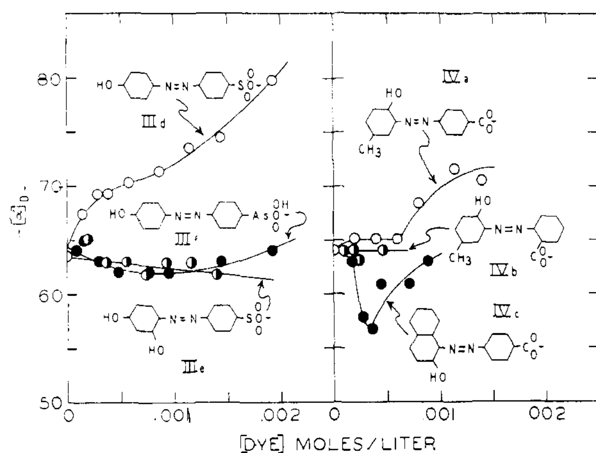

Fig. 5.—Specific rotation of 0.95% HSA as a function of the concentration of Group III and IV dyes in 0.15 *M* NaCl, 0.02 *M* phosphate buffer, *p*H 7.4.

Group IV (Fig. 5).—The first two dyes in this group are both strongly bound although the *para*-substituted carboxylate is again more effective. IV_b has no effect on the rotation in the highest measured concentration. (The intense light absorption of this complex prevented the use of higher concentrations.) The OH substituted naphthol derivative is effective both in binding and in changing the rotation.

The most important conclusion that can be drawn from these data is that the dimethylamino substituent in all cases lowers the levorotation, whereas the hydroxyl group in almost all cases raises it. An examination of the spectral shifts (Table I) resulting from the binding of the dye by the protein reveals a trend showing that those dyes that have a lowering effect on the levorotation also show a decrease in the wave length of maximum absorption, while a rise in the levorotation is associated with a slight increase or with no change in the absorption maximum.

Rotatory Dispersion.—The strong light absorption of the dyes unfortunately precluded the measurement of the rotatory dispersion below a wave length of 550 mμ. Measurements, however, were done in the presence of the dyes I_a (at two concentrations) and I_c from λ 725 mμ to λ 550 mμ, and the results are shown in Fig. 6. The dispersion in the case of the dye possessing the terminal OH group (I_c) appears to be normal as would be expected from the high levorotation at λ 589 mμ (−90°). The dispersion of HSA in the presence of the dye I_a is strongly anomalous, the anomalous character increasing with the dye concentration.

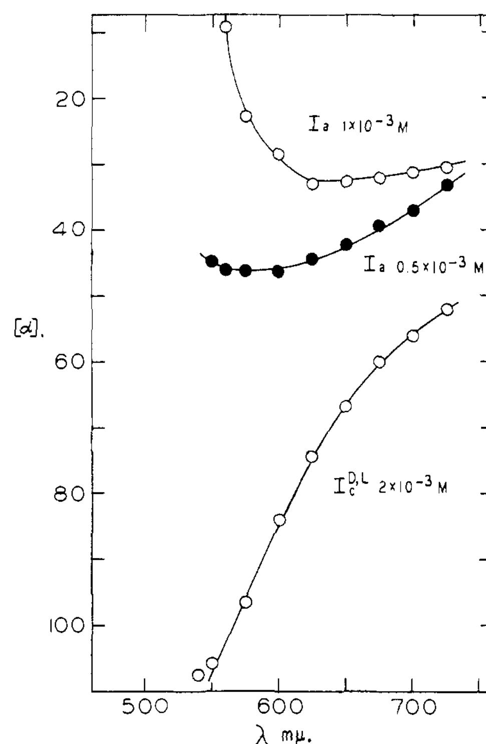

Fig. 6.—Rotatory dispersion of HSA in 0.5 × 10^{-3} *M* and 1 × 10^{-3} *M* I_a and 2 × 10^{-3} *M* I_c^{DL} dye, 0.15 *M* NaCl, 0.02 *M* phosphate buffer, *p*H 7.4.

Reversibility of Rotatory Changes.—Although the binding itself of azo dyes to serum albumin has been shown to be readily and entirely reversible, it appeared of interest to see whether the structural changes accompanying the interaction are also completely reversible, since a case to the contrary has been described by Hill and Briggs[10] for the interaction of the detergent *n*-octylbenzene-*p*-sulfonate with β-lactoglobulin. Two solutions containing 0.9% HSA and dyes I_c and I_d, respectively, in 1 × 10^{-3} *M* concentration at *p*H 7.4 were passed through columns of the anionic exchange resin Amberlite-400 (Cl^-) in order to remove the dyes. The subsequently measured optical rotation of the colorless effluents showed the restoration of the native value, indicating the complete reversibility of the structural changes.

Reduced Viscosity.—In contrast to the extensive rotatory changes caused by the interaction with these dyes the reduced viscosity shows only a slight change. This change in all measured cases consists in an increase of the reduced viscosity irrespective of the sign of the rotatory change, but does not exceed 10% of the native viscosity. In the case of dye I_c viscosities were measured over the entire concentration range investigated for the rotatory study. The results show an increase from 0.040 (no dye added) to 0.044 (2 × 10^{-3} *M* dye). The failure of the reduced viscosity to show an increase corresponding to the large rise in levorotation in this case is particularly significant for it indicates that the structural changes involved are not extensive in terms of the gross shape of the molecule.

(10) R. M. Hill and D. R. Briggs, This Journal, **78**, 1590 (1956).

TABLE I

BINDING DATA AND ABSORPTION MAXIMA[a]

HSA was 0.922% in 0.15 *M* NaCl and 0.02 *M* phosphate buffer, *p*H 7.4. BSA was 0.817% in 0.05 *M* phosphate buffer, *p*H 7.0. Initial dye concentration was 1 × 10^{-3} *M*.

Dye	Structure	Protein	Concn. of free dye, *M* × 10^4	*r*	Abs. max. of free dye, mμ	Abs. max. of bag contents, mμ
I_a	$(CH_3)_2N$—⬡—N=N—⬡—C(=O)—N(H)—C(H)(H)—CO_2^-	HSA	1.10	5.50	470	462
I_b^{DL}	⬡—N=N—⬡—C(=O)—N(H)—C(H)(C_6H_5)—CO_2^-	HSA	1.63	4.74	325	330
I_c^{DL}	HO—⬡—N=N—⬡—C(=O)—N(H)—C(H)(C_6H_5)—CO_2^-	HSA	0.766	5.96	357	364
I_d^D	$(CH_3)_2N$—⬡—N=N—⬡—C(=O)—N(H)—C(H)(C_6H_5)—CO_2^-	HSA	.404	6.48	470	440
I_d^D	$(CH_3)_2N$—⬡—N=N—⬡—C(=O)—N(H)—C(H)(C_6H_5)—CO_2^-	BSA	.590	6.22	470	440
I_d^L	$(CH_3)_2N$—⬡—N=N—⬡—C(=O)—N(N)—C(N)(C_6H_5)—CO_2^-	HSA	.312	6.60	470	440
I_d^L	$(CH_3)_2N$—⬡—N=N—⬡—C(=O)—N(H)—C(H)(C_6H_5)—CO_2^-	BSA	0.640	6.15	470	440
II_a	$(CH_3)_2N$—⬡—N=N—⬡—SO_3^-	HSA	.970	5.68	462	437
II_b	$(CH_3)_2N$—⬡—N=N—⬡—CO_2^-	HSA	.960	5.69	462	437
II_c	$(CH_3)_2N$—⬡—N=N—⬡ (ortho —CO_2^-)	HSA	2.63	3.34	428	414
III_a	⬡—N=N—⬡—CO_2^-	HSA	1.34	5.16	325	327
III_b	HO—⬡—N=N—⬡—CO_2^-	HSA	1.79	4.52	355	357
III_c	HO—⬡—N=N—⬡ (ortho —CO_2^-)	HSA	3.03	2.80	348	348
III_d	HO—⬡—N=N—⬡—SO_3^-	HSA	1.88	4.40	357	358
III_e	HO—⬡(OH)—N=N—⬡—SO_3^-	HSA	2.61	3.37	430	430
III_f	HO—⬡—N=N—⬡—AsO_3H^-	HSA	3.14	2.63	355	358
IV_a	⬡(OH)(CH_3)—N=N—⬡—CO_2^-	HSA	0.578	6.23	330	333
IV_b	⬡(OH)(CH_3)—N=N—⬡ (ortho —CO_2^-)	HSA	1.42	5.04	332	330
IV_c	naphthalene(OH)—N=N—⬡—CO_2^-	HSA	0.626	6.16	490	491

[a] The binding data for the dyes I_d^D and I_d^L were calculated from binding curves obtained earlier.[4,5]

Dye Binding.—In order to obtain a relative measure of dye–protein affinity the binding of all the dyes was determined with the same initial amount of dye (Table I). A comparison of the r values with the rotatory changes for the same dye shows some correlation between the magnitude of the rotatory change in the measured range and the strength of binding. Thus, members of Group I show the highest binding values and also the largest deviations from the native rotation. As pointed out before, the similarity of the rotatory curves of methyl orange and *p*-methyl red are paralleled by their similar binding properties. The weaker binding of the dyes *o*-methyl red, III_e and III_f are accompanied by small rotatory changes. However, the weak binding of III_c and the strong binding of IV_a and IV_c are not paralleled by corresponding rotatory changes.

Interpretation of Structural Changes.—While an unequivocal interpretation of the described rotatory changes is made difficult due partly to the limited range of the dispersion measurements and partly to the highly anomalous character of the dispersion curves, these changes can be best interpreted in terms of structural transformations of the protein molecule. That the dyes themselves are not the source of the rotatory changes can be concluded from the following: (a) Most of the dyes used have no asymmetric carbon atom. (b) Those that do show no measurable rotation in the concentration range employed. (c) Where both the D- and the L-forms of a dye were used (I_d), the rotatory changes occurred in the same direction.

The presence of the free dye in the solution has no effect on the rotation. This was shown by the change of $[\alpha]_D$ from $-46°$ to $-73°$ when 5×10^{-4} *M* I_d^D dye was displaced from its combination with HSA by the inclusion of 0.1 *M* sodium decyl sulfate in the solution. (Values around $-70°$ are characteristic for the HSA–detergent complex.)

Aside from the intrinsic residue contributions, probably the most important factor in determining the optical rotation of a protein in aqueous solution is the degree of helical organization of the polypeptide chains. The rotations observed in our studies correspond, therefore, to configurations varying in helical content. This view, although based primarily on measurements of $[\alpha]_D$ alone, is supported by the dispersion studies done on the two dyes causing rotatory changes of opposite sign. Thus $I_c^{D,L}$ with its property of raising the levorotation shows a normal dispersion corresponding to a low helical content, while I_a which has been most effective in decreasing the levorotation has a strongly anomalous dispersion indicating substantial helical content. The discussion of the rotatory changes caused by the various dyes clearly shows that these changes are dependent on the participation of the end groups of the dyes, either directly through interaction with the protein or indirectly by their effect on the stabilization of resonance forms of the dyes.

The organization of the polypeptide chain of the native albumin molecule is considered to be strongly dependent on interactions of the side chains. The small number of interacting dye molecules necessary to elicit the rotatory changes is particularly significant in this connection since it indicates a high degree of interdependence in the organization of the serum albumin molecule, such that a small configurational change at a few points in the molecule may bring about a reorganization of a large portion of the structure. The small difference in free energy between the helical and the randomly coiled forms of polypeptides in aqueous solutions recently calculated by Schellman[11] is consistent with our interpretation.

Configurational Adaptability.—The rotatory patterns obtained reflect sequences of structural changes, the extent and nature of the change depending on the dye used and the quantity of dye bound. They provide general support for the concept of configurational adaptability and direct experimental evidence of structural alteration of the protein molecule as a result of dye binding, as was proposed earlier.[4] The rotatory data indicate that the structural alteration may involve extensive changes on the level of the secondary structure.

The Role of Disulfide Bonds.—A significant feature of the structural changes described is their occurrence in the presence of intact disulfide bonds. That large structural transformations can occur without the breaking of these bonds in serum albumin was also shown by Yang and Doty[12] who found that the levorotation of native BSA was strongly reduced in a mixture of organic solvents. This observation indicates that the intramolecular position of the disulfide bonds is such as to permit considerable freedom of configuration for large segments of the peptide chain. The only slight changes in reduced viscosity, on the other hand, represent the limitations imposed by the disulfide bonds on the relative displacement of such segments. The large increase in reduced viscosity noted when serum albumin is reduced in the presence of sodium decyl sulfate shows the importance of these bonds in restricting the disorganization of the tertiary structure.[13]

Acknowledgment.—We are grateful to Dr. Walter Lobunez and Mr. Robert Marks for many helpful discussions and suggestions.

PHILADELPHIA, PA.

(11) J. A. Schellman, *Compt. rend. lab. Carlsberg, Sér. Chim.*, **29**, 230 (1956).

(12) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

(13) G. Markus and F. Karush, *ibid.*, **79**, 134 (1957).