with water to neutrality and finally evaporated after drying over sodium sulfate. Crystallization of the residue from acetone gave 375 mg. (32%) of 12-cycloethylenemercaptal of m.p. 270–280°. The analytical sample from acetone melted at 289–291° (Kofler).

*Anal.* Calcd. for $C_{29}H_{44}S_2O_4$: S, 12.31. Found: S, 11.98.

**22a,5α-Spirostan-3β-ol-11-one (Va) from the 12-Cycloethylenemercaptal (IX).**—IX (320 mg.) in 96% ethanol (55 ml.) was treated with Raney nickel (5 g., W-4), the mixture boiled for 3.5 hr., filtered hot and the nickel was washed with hot benzene. The combined solutions after concentration to dryness and crystallization of the residue from acetone–hexane yielded 160 mg. of crude Va, m.p. 207–214°. Recrystallization from the same solvent gave 100 mg. (38%) of Va, m.p. 219–224°, $[\alpha]^{20}D$ −30°. Acetylation of the mother liquors yielded 50 mg. (17%) of the 3-acetate Vb, m.p. 220–222°, or an over-all yield of 55%.

**22a,5α-Spirostane-3β,12β-diol-11-one 3-Monoacetate (IIc). A.**—A solution of IIa (2.0 g.) in glacial acetic acid (50 ml.) was boiled for 17 hours, cooled and the mixture poured into water, yielding 2.1 g. of crude product of m.p. 205–214°. Crystallization from methanol furnished 1.25 g. (57%) of IIc, m.p. 217–220°. The analytical sample derived from the same solvent exhibited m.p. 217–221° (Kofler 212–215°), $[\alpha]^{20}D$ −30°, $\nu_{max}^{CS_2}$ 1736 and 1708 cm.$^{-1}$ and hydroxyl group.

*Anal.* Calcd. for $C_{29}H_{44}O_6$: C, 71.28; H, 9.08. Found: C, 71.46; H, 8.99.

**B.**—A solution of IIa (2.0 g.) in glacial acetic acid (50 ml.) was treated with concentrated hydrochloric acid (0.5 ml.) and the mixture water precipitated after standing for 20 hours at room temperature. The crude solid (2.1 g., m.p. 208–218°) was crystallized from methanol to yield 1.35 g. (62%) of IIc, m.p. 216–221°, identical with the product obtained in Part A.

**22a,5α-Spirostane-3β,12β-diol-11-one 3-Acetate 12-Tosylate (IId).**—To a cooled solution of tosyl chloride (0.5 g.) in pyridine (3 ml.) was added 0.5 g. of IIc and the mixture was allowed to stand at room temperature for 22 hours, whereupon it was poured into ice-water and filtered, yielding 0.61 g. of crude tosylate of m.p. 123–129°. Crystallization from ether gave 0.45 g. (69%) of IId, m.p. 134–137°. The analytical sample, from ether, showed m.p. 140–145°, $\lambda_{max}$ 226 mμ, log ε 4.22.

*Anal.* Calcd. for $C_{35}H_{48}SO_8$: C, 67.30; H, 7.84; S, 4.98. Found: C, 67.11; H, 7.89; S, 4.99.

**Attempted Displacement of Tosylate IId with Iodide.**—Treatment of IId with sodium iodide in boiling anhydrous acetone for 24 hours resulted in slight decompositon of IId, but the product so obtained was halogen free. Operation in a sealed tube at 100°, while resulting in extensive decomposition of the tosylate, gave a product containing only a few per cent of halogen.

**Treatment of Tosylate IId with Raney Nickel.**—IId (0.5 g.) in 96% ethanol (25 ml.) was treated with W-4 Raney nickel catalyst (8 g.) and the mixture boiled for 19 hours. Removal of catalyst and concentration of the solvent gave a product with $\lambda_{max}$ 226 mμ, log ε 3.39 (some unchanged tosylate present), which on crystallization from methanol furnished 120 mg. of pure 22a,5α-spirostane-3β,12β-diol-11-one 3-monoacetate (IIc), m.p. 217–220°.

**Treatment of Tosylate IId with Lithium Aluminum Hydride.**—IId (0.5 g.) in anhydrous tetrahydrofuran (25 ml.) was treated with lithium aluminum hydride (0.5 g.) and the mixture boiled for 8 hours. After acetone decomposition of excess hydride and the usual work-up the crude product was crystallized from methanol yielding 0.2 g. of 22a,5α-spirostane-3β,11β,12β-triol, m.p. and mixture m.p. with an authentic sample[18] 261–263°.

MEXICO CITY, D.F.

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

# The Interaction of Optically Isomeric Dyes with Human Serum Albumin[1]

BY FRED KARUSH

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A study has been made of the interaction of human serum albumin with the optically isomeric forms of an anionic azo dye. By measurement of the competitive effects of structurally related colorless anions it is demonstrated that the combination for both forms of the dye involves an attractive three-point interaction. Since the same combining regions of the protein are utilized by the isomeric dyes it is concluded that these regions possess a high degree of configurational adaptability. Some selectivity is evident however as shown by the competitive effects of the isomeric forms of phenyl-(benzoylamino)-acetate. To account for the distinctive binding features of serum albumins it is assumed that for a considerable portion of the albumin molecule the interhelical attraction is relatively weak compared to other proteins such as γ-globulin. Substantial support for this view is found in the distinctive behavior of albumin in the denaturation studies of Kauzmann, *et al.*, and in the effect of low *p*H on this protein as reported by Yang and Foster. Low interhelical attraction permits the R substituents of the amino acid residues to assume various orientations relative to each other. These arrangements provide combining regions which can take on a variety of configurations. The binding of small molecules to these regions takes place within the protein molecule and causes further separation of the helices, a process which facilitates the binding of additional small molecules.

The combination of serum albumins with organic anions exhibits three distinctive features which serve to define the structural problems involved in this phenomenon. Firstly, the affinity between the protein and small molecule appears, in general, to be at least as great as that found, for comparable molecules, in the interaction between haptens and homologous antibodies and between enzymes and structural inhibitors. Secondly, organic anions of diverse molecular configurations are bound to the same combining regions of the protein as shown by their competitive relation. Thirdly, a single albumin molecule can bind many anions, in fact, as many as twenty, without any detectable irreversible alteration.

To account for the first two features of the complexing behavior of albumins we have utilized the concept of the configurational adaptability of the combining regions of the protein.[2] This means that these regions can assume a large number of configurations, arising from the variation of the relative positions of the amino acid side chains, and, therefore, can present more or less complementary configurations to a wide structural variety of small molecules. A recent investigation[3] of the interac-

(1) Presented in part before the Division of Biological Chemistry at the Los Angeles meeting of the American Chemical Society, March, 1953.

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

(3) F. Karush, Twenty-Fifth National Colloid Symposium, American Chemical Society, Ithaca, New York, June 18–20, 1951; *J. Phys. Chem.*, **56**, 70 (1952).

tion between optically isomeric azo dyes and bovine serum albumin, designed to provide clarification of this phenomenon, has yielded cogent support for this view. Furthermore, the same study has furnished evidence that complex formation is associated with a reversible structural alteration of the protein resulting in the availability of additional combining regions. The occurrence of such a change fits well conceptually with the observed multiple binding of albumins and is probably a necessary condition for it. As will be discussed below there is, in all likelihood, an essential connection between configurational adaptability of albumin and the suggested structural changes. This relation provides the basis for a unified interpretation of the distinctive binding properties of serum albumins. The notion that reversible structural alteration accompanies binding has also been stressed recently by Klotz, *et al.*,[4] and has found substantial support in the detailed denaturation studies of Kauzmann, *et al.*[5]

To ascertain the generality of the results found with bovine serum albumin we have investigated the interaction of human serum albumin with the optically isomeric forms of the anionic dye phenyl-(*p*-(*p*-dimethylaminobenzeneazo)-benzoylamino)-acetate. The results of this study, the details of which are described below, are similar to those found previously and serve to provide a more general interpretation of the phenomena under discussion.

## Experimental

The experiments reported here were carried out, for the most part, with a lyophilized preparation of human serum albumin kindly provided by the Commission on Plasma Fractionation and Related Processes. This material was prepared by the alcohol fractionation method and contains about 2% globulin. A value of 69,000 was used for the molecular weight of human albumin although this generally accepted figure may be somewhat too high.[6]

The optically isomeric dyes were the same as those described previously in our study of bovine albumin. The synthesis of the active dyes involved the use of the active forms of α-aminophenylacetic acid. Since the configuration of these amino acids has recently been established by Rudman, *et al.*,[7] the active dye previously designated *d*-$I_p$ will now be referred to as the L-$I_p$ dye and the *l*-$I_p$ dye as D-$I_p$. The optically active forms of phenyl-(benzoylamino)-acetic acid were prepared by benzoylation of the active amino acids.

The binding of the dyes by the protein was studied by the method of equilibrium dialysis under the same conditions of the earlier study.[2] The experiments were conducted at 25° in 0.0500 *M* phosphate buffer, *p*H 7.0, with an initial protein concentration of $3.00 \times 10^{-5}$ *M*.

## Results and Discussion

**The Binding Properties of the Protein.**—The experimental results for the binding of the isomeric dyes by human albumin are shown in Fig. 1 in the form of a plot of $r/c$ *vs.* $r$ where $r$ is the average number of dye ions bound per protein molecule at the free dye concentration $c$. The values of $r$ range from 1.7 to 19.3 and those of $c$ from $0.32 \times 10^{-5}$ *M* to $37.8 \times 10^{-5}$ *M*. The utility of this way of representing binding data has been indicated earlier. The substantial deviation of the binding curves from linearity can arise from a wide variation in the association constants reflecting configurational differences of the combining regions of the protein[2] or from interaction among the bound anions. However the theoretical electrostatic interaction between successively bound anions is insufficient, as has been shown in a similar case,[2] to account for the deviation.

To assess the full significance of the binding curves it is necessary to demonstrate that the isomeric dyes are bound to the protein sites by the attractive interaction of each of the three substituents of the asymmetric carbon atom (three-point interaction). This had been found to be true in the case of bovine albumin which has a somewhat lower affinity for the $I_p$ dyes. In Table I are given the results of experiments designed to provide evidence for an attractive three-point interaction. Measurements were made of the dye binding in the presence of two concentrations of several colorless organic anions structurally related to the dye. Two corresponding initial dye concentrations were used to yield low and high values of $r_0$ (see Table I). The competitive effects of these anions were evaluated and are expressed in terms of $r/r_0$, smaller values indicating greater inhibition. It is evident that the phenyl and benzylamino substituents separately and together contribute to the binding of the inhibitor. Little difference in this respect is found between the D- and L-isomers of phenyl-(benzoylamino)-acetate. Of particular importance is the observation that these substituents are contributory for both ranges of $r_0$ corresponding to portions of the binding curves differing markedly in their slopes. The contribution of the negative charge of the carboxyl group to the binding is evident from a comparison of phenylacetate and phenylacetamide and is in accord with the well established role of anionic groups in the binding of small molecules to albumins. On the basis of the above considerations, together with similar evidence for bovine serum albumin, we may conclude that the $I_p$ dyes are bound to human albumin by an attractive three-point interaction over most of the range of the binding curves and perhaps over the entire range.

The contributions which these substituents make to the binding reflect, partly at least, their hydrophobic character. It is not possible, therefore, to estimate the intrinsic contributions of these groups to the free energy of association of the inhibitors. This situation does not however alter the significance of the experimental results since they demonstrate that, in any case, the albumin molecule is able to provide regions whose contour is complementary to the inhibitor. If this were not the case then it would be expected that in general a substituent would either decrease binding by virtue of the steric hindrance it would introduce or else would have little influence on the binding. The latter effect would result if the substituent, in the bound state of the inhibitor, were located in such a manner as to remain surrounded by solvent molecules.

One of the most significant features of the binding curves is their close proximity to each other.

(4) I. M. Klotz and J. Ayers, *Faraday Soc. Discs.*, **13**, 189 (1952).

(5) W. Kauzmann and R. B. Simpson, THIS JOURNAL, **75**, 5154 (1935); H. K. Frensdorff, M. T. Watson and W. Kauzmann, *ibid.*, **75**, 5167 (1953).

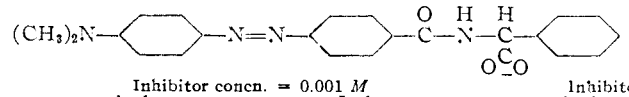
(6) B. W. Low, *ibid.*, **74**, 48 (1952).

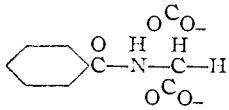
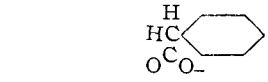
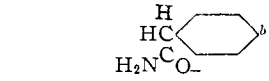
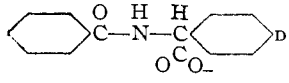
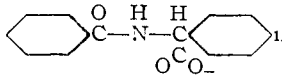
(7) D. Rudman, A. Meister and J. P. Greenstein, *ibid.*, **74**, 551 (1952).

TABLE I

THE INHIBITORY EFFECT OF ORGANIC ANIONS ON THE BINDING OF D-$I_p$ AND L-$I_p$ DYES BY HUMAN SERUM ALBUMIN

$3 \times 10^{-5}$ *M* albumin, 0.05 *M* phosphate, *p*H 7.0, 25°[a]



| Inhibitor | Inhibitor concn. = 0.001 *M*: D-$I_p$ dye, Initial concn. = $9.53 \times 10^{-6}$ *M* | | | Inhibitor concn. = 0.001 *M*: L-$I_p$ dye, Initial concn. = $9.86 \times 10^{-6}$ *M* | | | Inhibitor concn. = 0.005 *M*: D-$I_p$ dye, Initial concn. = $38.5 \times 10^{-6}$ *M* | | | Inhibitor concn. = 0.005 *M*: L-$I_p$ dye, Initial concn. = $39.3 \times 10^{-6}$ *M* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $c \times 10^5$ | $r_0$ | $r/r_0$ | $c \times 10^5$ | $r_0$ | $r/r_0$ | $c \times 10^5$ | $r_0$ | $r/r_0$ | $c \times 10^5$ | $r_0$ | $r/r_0$ |
| H | 0.599 | 2.83 | 0.976 | 0.667 | 2.87 | 0.986 | 6.43 | 8.43 | 0.994 | 6.06 | 8.97 | 0.964 |
| HCH | .600 | 2.84 | .972 | .674 | 2.88 | .980 | 6.36 | 8.40 | 1.004 | 6.01 | 8.93 | .972 |
|  | .850 | 3.43 | .753 | .860 | 3.33 | .808 | 6.91 | 8.67 | 0.889 | 6.92 | 9.56 | .841 |
|  | .896 | 3.52 | .725 | .932 | 3.49 | .756 | 7.28 | 8.87 | .840 | 7.38 | 9.84 | .785 |
|  [b] | .620 | 2.90 | 1.01 | .682 | 2.90 | .970 | 5.48 | 7.88 | .952 | 5.35 | 8.38 | .915 |
|  D | 1.400 | 4.44 | 0.491 | 1.266 | 4.13 | .581 | 8.60 | 9.44 | .689 | 8.38 | 10.34 | .678 |
|  L | 1.318 | 4.32 | 0.517 | 1.351 | 4.28 | .542 | 9.17 | 9.67 | .639 | 8.92 | 10.57 | .646 |

[a] $c$ = free dye concentration at equilibrium; $r$ = moles of dye bound per mole of protein in presence of inhibitor; $r_0$ = moles of dye bound per mole of protein in absence of inhibitor. [b] Somewhat different initial dye concentrations were used in this case.

This means not only that the D-$I_p$ and L-$I_p$ dyes are bound with almost equal affinities but that the same combining regions of the protein are utilized for both dyes. This conclusion follows from the position of the DL-curve relative to the other two curves. If different sites were involved then, as has been shown before,[3] the DL-curve would lie above the observed one, the relative divergence increasing with larger $r$. Since the isomeric dyes are bound through an attractive three-point interaction, it is clear that the combining regions of the protein must possess a high degree of configurational adaptability in order that they may present the different complementary configurations required for these interactions. The distinctive character of serum albumins in this respect can, perhaps, be most clearly seen from the contrasting behavior of antihapten antibody ($\gamma$-globulin). Rabbit antibodies prepared against the D- and L-forms of $I_p$ hapten, phenyl-(*p*-azobenzoylamino)-acetate, exhibit an almost complete discrimination between the isomers.[8] In addition, purified anti-D-$I_p$ antibody will bind the D-$I_p$ dye and alter its absorption spectrum but will not do so with the L-$I_p$ dye.[9] Since the association constants for the $I_p$ dye of human albumin and the homologous antibody appear to be of the same order of magnitude it is evident that the closeness of fit of the antibody combining region which gives rise to its selectivity must be duplicated by the albumin molecule. In contrast to antibody, however, the same regions of the albumin molecule can provide this closeness of fit for both the D- and L-forms of the $I_p$ dye.

(8) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **48**, 315 (1928).

(9) F. Karush, *Federation Proc.*, **12**, 448 (1953).

That the albumin does exercise some selectivity between the isomeric dyes is evident from the fact that the D- and L-curves are not identical. The selectivity, however, is not consistent since at low values of $r$ the D-$I_p$ dye is bound more strongly than the L-$I_p$ dye whereas at larger $r$ the reverse is true. This selectivity also appears in the behavior of the optically isomeric inhibitors to be discussed below.

The binding of the $I_p$ dyes by human serum albumin differs from that by bovine serum albumin in that the binding of the L-$I_p$ dye by the latter gave a sigmoidal curve. The shape of this curve together with other considerations provided the grounds for our assertion that the binding of the first few dye molecules made available additional combining regions.[3] It was also shown that if this is the case for the L-$I_p$ dye it must also be true for the D-$I_p$ dye although in the latter case the binding curve showed only a decreasing negative slope with increasing $r$, as with the curves for human albumin. In the case of human albumin also the same interpretation of the effect of the initially bound anions on the binding of additional molecules appears applicable.

In order to provide a mathematical formulation for this picture of sequential binding we consider the highly simplified situation of a protein molecule capable of multiple binding by a mechanism in which complex formation with the first small molecule makes available a second site, the occupancy of which in turn provides another region, etc. If this process occurs by a fixed sequence then it is easily demonstrated by mass action considerations that the fraction ($\alpha_n$) of any group of sites, identified by a particular ordinal position ($n$) in this sequence,

which is combined at a particular free equilibrium concentration $c$ is given by

$$\alpha_n = \prod_{i=1}^{n} \left(\frac{K_i c}{1 + K_i c}\right) \quad (1)$$

and

$$r = \sum_n \alpha_n = \sum_n \left[\prod_{i=1}^{n} \left(\frac{K_i c}{1 + K_i c}\right)\right] \quad (2)$$

For the case that the $K_i$'s are equal and the number of sites per protein molecule is infinite equation 2 reduces to $r = cK$ and a plot of $r/c$ *vs.* $r$ yields a straight line parallel to the horizontal axis. It is interesting to note that the first part of the curve describing the binding of the L-$I_p$ dye by bovine albumin approximates this situation.[3] Although we have not solved (2) for a simple analytical relation between $r$ and $c$ based on any other distribution of the $K_i$'s it is clear from this equation that a $r/c$ *vs.* $r$ plot can yield either increasing or decreasing values of $r/c$ with increase of $r$ depending on the kind of distribution.

It is apparent from the binding curves for the two proteins that human albumin possesses a greater affinity for the dyes than does bovine albumin. The D-$I_p$ dye, for example, is bound about twice as strongly by the former as by the latter. Although this difference between the proteins depends somewhat on the manner of their preparation it undoubtedly reflects an intrinsic structural dissimilarity between them. The superiority of human albumin in the binding of several azo dyes also has been observed by Klotz, *et al.*[10] It is worthy of note that for both proteins not only do the binding curves cross but also the relative binding of the isomers is qualitatively alike for large and small $r$.

The importance of the preparative history of the protein is evident from the fact that the binding curves for the $I_p$ dyes found with a sample of crystallized human albumin were significantly higher than those shown in Fig. 1 in the range of small $r$ (2 to 5). On the possibility that this difference might be due to a dialyzable impurity reversibly bound to the amorphous albumin, a portion of this sample was dialyzed in the cold room for a few days against several changes of distilled water. The protein solution was lyophilized and binding curves established. However, no difference in the binding behavior was found as a result of the dialysis treatment. The dependence of the binding behavior of albumins on their mode of preparation was also noted by Klotz, *et al.*[10]

**Inhibition by Optically Isomeric Competitors.**—The differences in the binding curves (Fig. 1) of the $I_p$ dyes demonstrate that the combining regions of the protein exercise a selectivity between the isomeric dyes. For small values of $r$ the D-$I_p$ dye is bound more strongly than the L-$I_p$ dye whereas for larger values the reverse is true. This selectivity can be explored in a more effective manner by the use of optically isomeric inhibitors. For this purpose we have evaluated the competitive effect of the isomeric forms of phenyl-(benzoylamino)-acetate on the dye binding for small and intermediate values of $r$ using inhibitor concentrations of $1.00 \times 10^{-3}$ $M$ and $5.00 \times 10^{-3}$ $M$, respectively. The results are presented in Figs. 2 and 3 in the form of plots of $r/r_0$ *vs.* $c$ where the symbols have the same meaning as in Table I. The curves of Fig. 2, for which $r_0$ varies from 3 to 5, show that each isomeric dye is most effectively displaced from the protein by the inhibitor with the same configuration. It follows then that some of the combining regions involved in this range of $r_0$ form slightly firmer complexes with one isomeric inhibitor than with the other while others exhibit the alternative preference. Additional confirmation of this interpretation is furnished by the fact that the DL binding curve is significantly higher for small $r$ than the other curves since such a difference is to be expected only when the selectivity described above is operative. It is quite obvious that the present information does not allow a decision as to whether this selectivity occurs among the sites of a single protein molecule or whether it reflects a variation from one molecule of albumin to another.

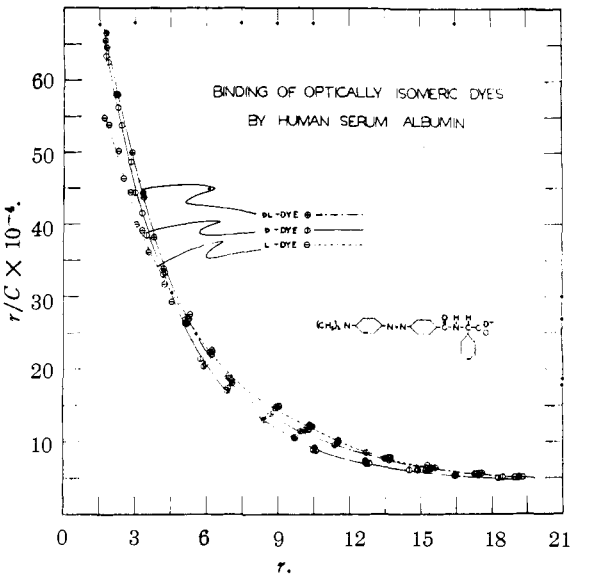



Fig. 1.—Binding curves for combination of isomeric dyes with human albumin at 25° in 0.05 $M$ phosphate buffer, $p$H 7.0, protein concentration = $3.00 \times 10^{-5}$ $M$.

The variations in the effectiveness of the inhibitors represent relatively small differences in their interaction with the protein, as is apparent from the proximity of the curves of Fig. 2. The extrapolation of these curves to zero dye concentration yields average association constants for the binding of the inhibitors to the combining regions of the albumin which are involved at small $r$. The values so obtained from the four curves range from $3 \times 10^3$ to $4 \times 10^3$. In view of the three-point attachment of these inhibitors to the protein the closeness of the inhibition curves and the association constants must be attributed to the configurational adaptability of the protein.

A different behavior of the isomeric inhibitors is found at higher dye concentrations. This is seen in Fig. 3, for which $r_0$ ranges from 7 to 12, since for $c$ greater than $5 \times 10^{-5}$ $M$ the L-inhibitor is more ef-

(10) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, THIS JOURNAL, 56, 77 (1952).

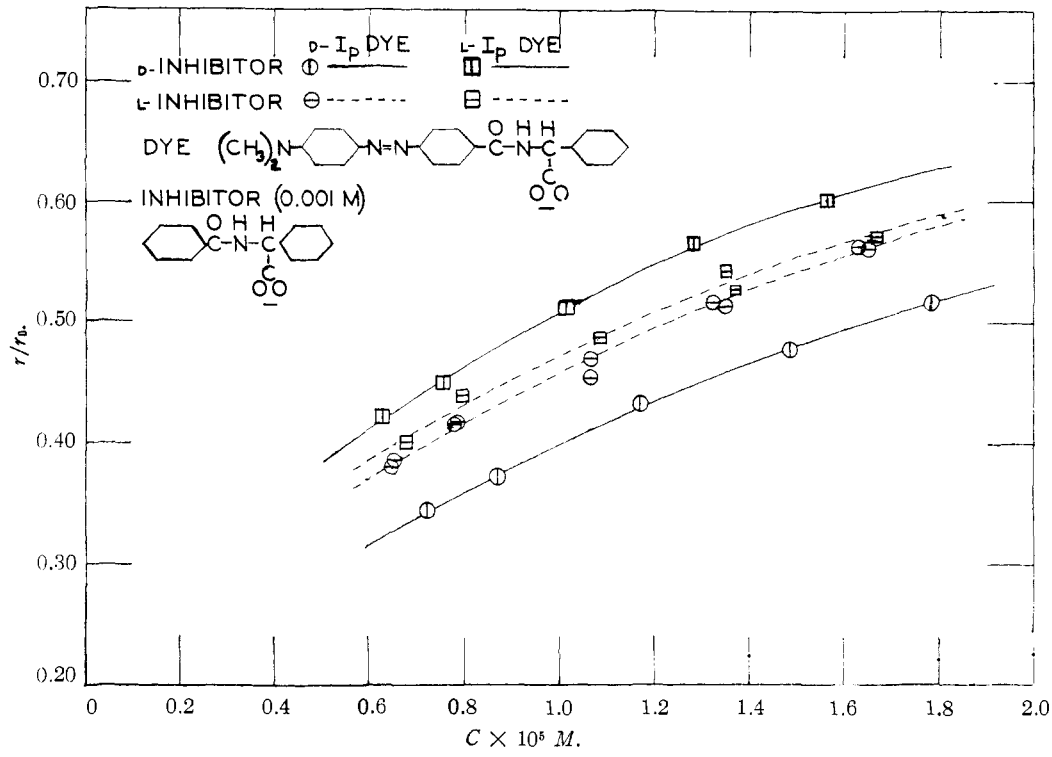



Fig. 2.—The inhibitory effects of the isomeric forms of phenyl-(benzoylamino)-acetate on the binding of the isomeric $I_p$ dyes for small values of $r_0$.

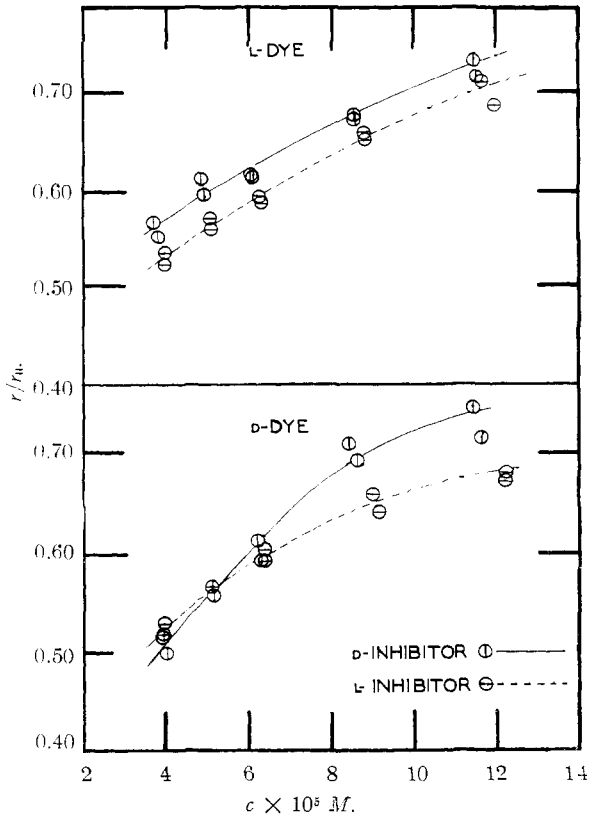



Fig. 3.—The inhibitory effects of the isomeric forms of phenyl-(benzoylamino)-acetate on the binding of the isomeric $I_p$ dyes for intermediate values of $r_0$. Concentration of inhibitor is $5.00 \times 10^{-3}$ $M$.

fective than the D-inhibitor for both isomeric forms of the $I_p$ dye. This result implies that the additional combining regions of the proteins which participate in this range of $r_0$ exercise a more or less consistent selectivity in favor of the L-inhibitor. The stronger binding of the L-$I_p$ dye than of the D-$I_p$ dye in the range of $r$ of 7 to 12, in the absence of inhibitor, and the intermediate position of the DL-curve are consistent with this selectivity. Indeed, these relations are to be expected if it is assumed that the contribution of the *p*-dimethylaminobenzeneazo group to the binding is about the same for the isomeric dyes.

**The Effect of *p*H.**—In Fig. 4 are shown the results of binding experiments carried out at *p*H's 6, 7 and 8 with 0.05 $M$ phosphate buffers in the range of small $r$. It is evident that the binding diminishes with increase in *p*H as would be expected from the increased electrostatic repulsion between the dye anion and the protein. This result agrees with earlier observations[11] of the effect of protein charge on the binding of methyl orange by bovine serum albumin. The experiments with methyl orange were performed in the absence of buffer using the method of partition analysis.

In addition to the electrostatic effect there appears to be a structural alteration of the protein associated with the *p*H region 6 to 8. This effect can be inferred from the change with *p*H in the relative binding of the isomeric dyes. This change consists of an increasing divergence between the curves of the $I_p$ dyes as the *p*H decreases. Nevertheless, each pair of curves crosses at a value of $r$ between 4 and 5, a result which serves to demonstrate that the

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

structural alteration which is *p*H dependent is not the same as that which we attribute to the binding of the dye. By the same token we also conclude that the latter alterations, those giving rise to sequential binding, are not simply the result of changing the charge of the protein and thereby affecting structural features which depend on intramolecular electrostatic repulsion. The conclusion that bovine albumin also undergoes a structural change in the *p*H range 6 to 8 was reached in our earlier study.[11] Independent evidence that human albumin undergoes a structural change in the *p*H region near 7 has been provided by Klotz, Burkhard and Urquhart.[10] These authors showed that the absorption spectrum of bound methyl orange changes substantially in this *p*H region.

Recently, on the basis of binding studies with neutral azo dyes, Klotz and Ayers[12] have concluded that the intrinsic binding affinity of bovine and human albumin increases when the *p*H is raised from 6.9 to 9.2. However, since different buffers were used, phosphate at *p*H 6.9 and glycine at *p*H 9.2, and since their competitive effects on the binding of the neutral molecules are not adequately known, it appears that further clarification of this problem is required. The results of Fig. 4 are not very useful in this connection because it is not possible to make reliable corrections for the electrostatic repulsion due to the different protein charges and for the competitive effects of the monovalent and bivalent phosphate anions, the ratios of which are greatly different at the three *p*H's used.

**Configurational Adaptability and Interhelical Interaction.**—From our earlier study of the binding of the $I_p$ dyes by bovine serum albumin we concluded that there is a reversible structural alteration of albumin associated with the binding process. It was also suggested that a portion of the entropy increase invariably found in the binding of anions was due to the rupture of intramolecular protein bonds. As noted above there appears to be an intrinsic relation between this structural effect and the configurational adaptability of albumin. For the sake of concreteness in formulating this relation we may assume the Pauling–Corey $\alpha$-helix for serum albumins.[13] Although this assumption is not essential for our purpose, evidence in its favor has been provided by Riley and Arndt.[14] We further picture the albumin molecule to be made up of close-packed rods ($\alpha$-helices) generally similar to the arrangement deduced for the hemoglobin molecule.[15] The stability of this close packed structure depends on the attractive interactions between the substituents of the neighboring helices.

We now make the essential assumption that for a considerable portion of the albumin molecule the interhelical attraction is relatively weak compared to that of other kinds of proteins, *e.g.*, $\gamma$-globulin. Thus the helices of albumin could undergo some separation from each other under conditions which did not suffice for other proteins and, furthermore, the energy of activation for such a process would be less

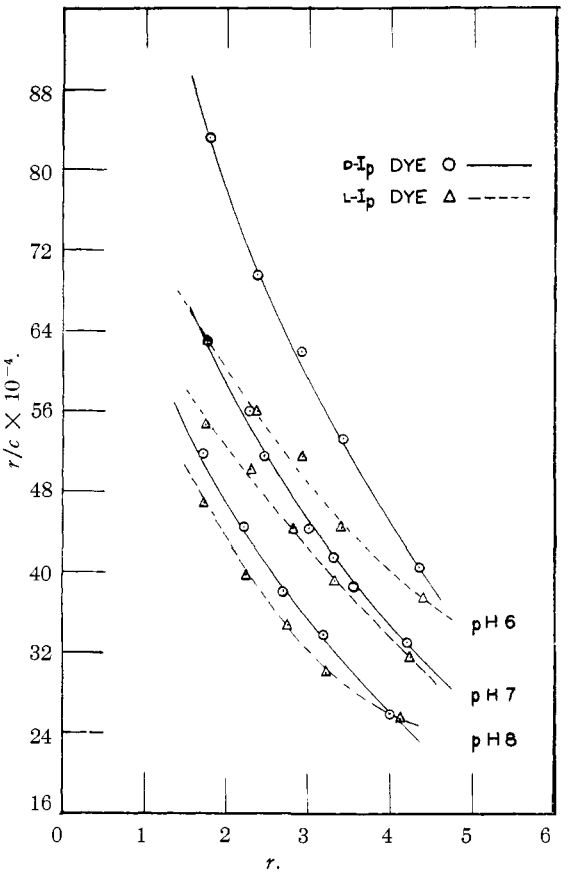



Fig. 4.—The effect of *p*H on the binding of the isomeric $I_p$ dyes in 0.05 *M* phosphate buffer.

for albumins. The recent denaturation studies of Kauzmann, *et al.*,[4] provide striking support of this view. These investigators found that in the urea denaturation of bovine serum albumin there is an immediate change in the optical activity and viscosity. In the case of egg albumin, on the other hand, these properties change much more slowly starting with the values characteristic of the native protein. The bearing of these differences on the distinctive binding behavior of serum albumins was emphasized by these authors who suggested that the ease of denaturation of serum albumin was due to a lack of rigidity of the protein molecule which also made possible configurational adaptability. Additional evidence in favor of our assumption of low interhelical attraction for albumin comes from the results of Yang and Foster[16] on the changes in the intrinsic viscosity and optical rotation of bovine albumin associated with acid binding. These authors demonstrated that this protein, unlike ovalbumin, undergoes large changes in these properties when the *p*H is reduced from the isoelectric region to about 2. At the latter *p*H the albumin molecule carries a positive charge of approximately 100. Because of the large coulombic repulsion Yang and Foster suggest that, as in the case of polymeric electrolytes, there is a considerable swelling of the molecule, the extent of which is dependent on the ionic strength of the medium.

The dependence of configurational adaptability

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(13) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci.*, **37**, 205 (1951).
(14) D. P. Riley and U. W. Arndt, *Nature*, **169**, 138 (1952).
(15) W. L. Bragg and E. R. Howells, *Acta Cryst.*, **5**, 136 (1952).
(16) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

on interhelical separation stems from the consideration that when the R substituents of the amino acid residues are freed from the spatial constraints associated with close-packing of the helices, these substituents are able to assume various positions due to the rotation possible about the single bonds which link them to the polypeptide chain. This variability makes possible a variety of configurations consisting of the various ways the substituents can be oriented with respect to each other. Such groups of substituents comprise the potential combining regions of the protein since these groups can provide the more or less complementary configurations necessary to bind strongly various organic structures.

The binding of the small molecule, we suggest, takes place not on the surface of the protein molecule but within it, in the regions between the helices, and involves substituents contributed by two or three adjoining helices. The introduction of a molecule into the protein leads to a further separation of the helices thereby facilitating the binding of additional molecules. In this way a large number of molecules can be bound to a single protein molecule and an explanation is thus provided for the interaction of bound molecules as well as their stabilizing effect against denaturation. The stabilization would be a consequence of the strengthened linkage between neighboring helices provided by the bound molecules.

On the basis of the above considerations it would be expected that proteins other than serum albumins would acquire non-specific binding properties if their interhelical attraction could be reduced. Such a reduction would have to be effected, of course, under conditions which did not preclude binding because of other factors, such as the competition due to a high concentration of urea.

Several authors[17,18] have recently called attention to the fact that complex formation with protein involves the transfer of a small molecule from a region of high dielectric constant to one of low dielectric constant. This fact would favor the binding of anions such as chloride and anionic groups such as carboxyl when these are involved in electrostatic interaction with positively charged groups of the protein, as well as the binding of organic molecules generally.

There is little reason to doubt that this factor is an essential feature of the binding process, not only for albumins but also for the specific interactions of antibodies and enzymes. It is evident, however, that this consideration is applicable to proteins generally and cannot, therefore, serve to account for the distinctive properties of serum albumins.

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(18) J. A. Schellman, *J. Phys. Chem.*, **57**, 472 (1953).

It is instructive to compare the specific binding of anti-hapten antibodies and the non-specific binding of albumins with the solvent properties of crystalline solids and liquids, respectively. That there is a parallelism between the specificity of such antibodies and the isomorphism of crystals was first pointed out by Erlenmeyer and Berger.[19] They demonstrated that the $-PO_3H^-$ and $-AsO_3H^-$ groups are immunologically equivalent but different from $-SbO_3H^-$ in accordance with the isomorphism of salts of phosphate and arsenate and the crystalline dissimilarity of antimonate. In the case of organic molecules the same relationship is shown by the fact that mixed crystals are formed by *p*-dibromobenzene and *p*-bromotoluene in accordance with the immunological equivalence of *p*-bromoaniline and *p*-toluidine.[20] On the other hand the relative non-selectivity of liquids as solvents in contrast to their crystalline forms arises from the ability of the solvent molecules to rearrange readily their relative positions to conform to the configuration of the solute molecule. This provides a complementary region which serves to maximize the attractive interaction between the solute and solvent. The configurational adaptability which is involved in this process is possible because of the small energy required for the relative displacement of the solvent molecules, analogous to the ready displacement of the R groups of the amino acid residues.

In an interesting attempt to account for albumin binding in terms of amino acid composition Klotz *et al.*,[4,21] have emphasized the importance of the relatively low content of hydroxyamino acids in serum albumins as compared to bovine γ-globulin, for example. It is suggested that the hydroxyl groups form hydrogen bonds preferentially with the carboxyl groups rather than with the cationic groups. In the case of serum albumins, this preference of the hydroxyl-containing residues and their small number result, it is proposed, in a less rigid molecule and render the cationic groups more readily available than in other proteins. In terms of this picture the cationic groups might be expected to be contained in the regions of the protein of low interhelical attraction where configurational adaptability is possible. On this basis the much greater binding of organic anions compared to organic cations could be explained as well as the competition between neutral molecules and organic anions.

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