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

The Interaction of Purified Antibody with Optically Isomeric Haptens[1,2]

BY FRED KARUSH

RECEIVED MARCH 27, 1956

An investigation has been made of the interaction between purified antibody specific for the D- and L-forms of phenyl-(*p*-azobenzoylamino)-acetate and the homologous azo dyes D- and L-phenyl-(*p*-(*p*-dimethylaminobenzeneazo)-benzoylamino)-acetate. The azohapten–antibody combination was studied by the method of equilibrium dialysis at two temperatures. The inhibitory effect of several structurally related haptens has also been evaluated. The data for six different antibody preparations allow the unambiguous extrapolation of their binding curves to yield, consistently, a value of 2.0 (±5%) for the antibody valence. From the temperature dependence of these curves it is concluded that ΔS° for the association is practically zero. The values of ΔF° for the reaction at 25° range from −6.7 to −7.8 kcal./mole of hapten. The binding curves also serve to demonstrate the heterogeneity of the combining regions of the antibody with respect to their affinity for the azohapten. This heterogeneity can be adequately described by a Gauss error function in the free energy of binding. An explanation is provided for the differences in the thermodynamic results for the binding of the azohaptens by antibody and serum albumin. The markedly different spectral shifts effected by these proteins can also be accounted for in a manner consistent with this explanation. The inhibition results indicate that the combining region of the antibody is probably not larger than would be required to make contact with the entire azohapten. These results also show that the α-phenyl group and the benzoylamino group, substituents on the asymmetric carbon atom of the azohapten, contribute 4 and 2.4 kcal., respectively, to the free energy of binding. The anionic group also contributes substantially, but only a maximum value of about 2 kcal. can be estimated in this case.

Although much attention has been devoted to the specific reaction between antigen and antibody over the past several decades, little information is available regarding the thermodynamic aspects of this process. Perhaps the main reasons for this deficiency in our knowledge are to be found (1) in the absence of any general method for the specific purification of antibody and (2) in the fact that the antigen–antibody reaction cannot generally be studied in a homogeneous system under conditions where the concentrations of all the components can be measured.

In the face of these experimental difficulties the use of anti-hapten antibody offers decisive advantages in the investigation of the thermodynamics of the specific reaction. Antibody prepared against known haptenic structures can be purified readily since the specific precipitate containing the anti-hapten antibody is easily dissociated and solubilized with the aid of an appropriate hapten. The specific combination between the purified antibody and the homologous hapten to form a soluble complex can then be studied in a homogeneous system under equilibrium conditions.

One of the most useful methods for this purpose is the technique of equilibrium dialysis. The first application of this method to antibody binding was made by Marrack and Smith in 1932.[3] These authors were able to demonstrate the specific combination of the azo dye *p*-phenylazobenzenearsonic acid with the globulin fraction of rabbit antisera prepared against arsanilic acid coupled to horse serum. Shortly thereafter, Haurowitz and Breinl[4] studied the binding of a less strongly bound hapten, arsanilic acid, to the same kind of antisera. More recently Eisen and Karush,[5] using purified antibody specific for the *p*-azobenzenearsonic acid group, studied its binding of the homologous hapten *p*-(*p*-hydroxyphenylazo)-benzenearsonic acid at room temperature. These experiments yielded an association constant of 3.5×10^5, corresponding to $\Delta F^0 = -7.7$ kcal. per mole hapten and an antibody valence of 2 (±10%). This method has also been effectively applied by Carsten and Eisen[6] to the investigation of the specific reaction between a nonionic haptenic group and its homologous rabbit antibody. In this work the antibody was formed against dinitrophenyl-BγG (bovine γ-globulin), and the haptens used were dinitroaniline and ε-dinitrophenyllsine.

In this paper we present the results of a detailed investigation of antibody–hapten interaction. The haptenic groups selected for study were the optically isomeric forms of phenyl-(*p*-azobenzoylamino)-acetate, henceforth referred to as D-I_p and L-I_p. It had previously been demonstrated by Landsteiner and van der Scheer[7] that the D- and L-haptenic groups gave rise to antibodies which readily distinguished between the isomers. It appeared therefore that these isomers would be particularly suitable for the quantitative evaluation of cross-reactivity. In addition we have employed them to investigate the possibility of the formation of antibodies with dual specificity. This work will be described elsewhere.

Experimental

Materials.—The preparation of the enantiomorphic forms of the haptenic groups in a high state of optical purity was made possible by the method of enzymatic resolution described by Birnbaum, *et al.*[8] This procedure is based on the asymmetric hydrolysis of N-acylated racemic amino acids by an enzyme preparation from hog kidney termed acylase I. The preparation by this method of the optically active forms of α-aminophenylacetic acid, the starting material for the synthesis of the hapten, was reported by Rudman,

(1) Presented in part before the Division of Biological Chemistry at the Minneapolis meeting of the American Chemical Society, September, 1955.

(2) These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the Children's Hospital of Philadelphia, NR 120-099 and by a research grant (G-4139) from the National Institutes of Health, Public Health Service. Reproduction in whole or in part is permitted for any purpose of the United States Government.

(3) J. Marrack and F. C. Smith, *Brit. J. Exptl. Path.*, **13**, 394 (1932).

(4) F. Haurowitz and F. Breinl, *Hoppe-Seyler's Z. physiol. Chem.*, **214**, 111 (1933).

(5) H. N. Eisen and F. Karush, THIS JOURNAL, **71**, 363 (1949).

(6) M. E. Carsten and H. N. Eisen, *ibid.*, **77**, 1273 (1955).

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

(8) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

et al.[9] The chloroacetyl derivative of DL-α-aminophenylacetic acid was prepared by the acylation of the racemic amino acid with chloroacetic anhydride at *p*H 12 in an ice-bath. The product was purified by solution in ethyl acetate and evaporation to dryness. The isomeric amino acids were obtained from this material in accordance with the method of Birnbaum, *et al.*[8] It is of interest to note that this enzymatic method also serves to identify the D- and L-enantiomorphs.

The optical purity of the amino acid enantiomorphs has been evaluated by the use of L- and D-amino acid oxidases following the procedure described by Meister, *et al.*[10] This method makes possible the determination of the level of contamination of an amino acid by its optical isomer to less than 1 part in 1000. The analyses were carried out with 1000 μmoles of one or the other of the isomers per Warburg flask. For the detection of the L-α-aminophenylacetic acid 30 mg. of rattlesnake venom (Ross Allen's Reptile Institute) were used to provide the L-amino acid oxidase and for the D-contaminant 20 mg. of a D-amino acid oxidase preparation from hog kidney (Worthington). On the basis of the oxygen consumption observed with the flasks to which 1 and 2 μmoles of the susceptible substrate were added, it is concluded that the optical purity of each isomer is greater than 99.9%.

The isomeric amino acids were used to prepare the optical isomers of phenyl-(*p*-aminobenzoylamino)-acetic acid, the intermediates required for coupling to protein, following the procedure of Ingersoll and Adams.[11] This preparation involves the synthesis of phenyl-(*p*-nitrobenzoylamino)-acetic acid, the isomers of which were also used in the inhibition experiments to be described later. Optical rotations of the amino compounds measured at room temperature in 1 *N* HCl at a concentration of 6.7% gave $[\alpha]_D$ +92.6° for the L-derivative and $[\alpha]_D$ −93.2° for the D-compound. The values reported for $[\alpha]^{20}D$ in the same solvent by Ingersoll and Adams[11] are 93.63° and −93.75° for the L- and D-products, respectively.

The benzoyl derivatives of the amino acid enantiomorphs were also prepared for use in the inhibition experiments by the reaction with benzoyl chloride at *p*H 11–12 in an ice-bath. The optical rotations were determined at room temperature at a concentration of 2% in 1 *N* KOH. These gave $[\alpha]_D$ +88.8° for the L-isomer and $[\alpha]_D$ −89.7° for the D-isomer.[12]

The binding experiments were carried out with the optically isomeric forms of the azohapten phenyl-(*p*-(*p*-dimethylaminobenzeneazo)-benzoylamino)-acetate. The preparation and absorption properties of these dyes, called D-I_p dye and L-I_p dye, have been described previously.[13] The absorption spectrum is reproduced in Fig. 4. These dyes were synthesized with intermediates derived from amino acid enantiomorphs resolved by a non-enzymatic procedure. The optical purity of the dyes is to be judged from the specific rotations of the isomeric intermediates, D- and L-phenyl-(*p*-aminobenzoylamino)-acetic acid, used in their preparation.[13] In view of this the presence of an isomeric contaminant to the extent of 1% cannot be excluded. However, for the purposes of the binding studies, this amount of optical impurity would introduce a negligible error.

The immunizing antigens were prepared by diazotizing D- and L-phenyl-(*p*-aminobenzoylamino)-acetic acid in the conventional way and coupling to bovine γ-globulin (Armour, fraction II) to yield D-I_p–BγG and L-I_p·BγG. The usual procedure is to diazotize 3 millimoles of the intermediate and to add it dropwise to a solution of 4 g. of BγG in 200 ml. of 0.15 *M* NaCl, at 0–5°, maintained at *p*H 9 by simultaneous addition of 1 *N* NaOH. After addition of the diazonium salt the solution is kept at these conditions for about 1 hr. and then placed in the cold room overnight. During this period the *p*H drops to between 8 and 7.5. The *p*H is adjusted to 7.0 and the solution is dialyzed for several days in the cold room against 6 liters of 0.15 *M* NaCl changed daily. This procedure has been adopted to minimize denaturation and the indications are that this has indeed been avoided.

The number of azo groups per protein molecule has been deduced indirectly from the absorption properties of the azoprotein. In 0.15 *M* NaCl, 0.02 *M* PO_4, *p*H 7.4 D-I_p-BγG has an absorption maximum at λ 341 mμ with an optical density, for a 1 cm. path-length, of 0.0276 at 1.00 μg. protein N/ml. For purposes of calculation we have assumed that the molar extinction coefficient of the haptenic group of the azoprotein is equal to that for the related dye phenyl-(*p*-benzeneazobenzoylamino)-acetic acid. In 0.15 *M* NaCl, 0.02 *M* PO_4, *p*H 7.4, this dye has an absorption maximum at λ 325 mμ and a molar extinction coefficient of 2.45×10^4. On this basis it is computed that there are, on the average, 27 haptenic groups per protein molecule based on a molecular weight of 164,000 for the azoprotein (156,000 for the protein + 8,000 contributed by the haptenic groups).

The immunizing antigens are injected in the form of alum precipitates. It has been observed that the azoproteins can be rendered insoluble at *p*H 7.4 by the use of a weight ratio of protein to alum of 2.7. A typical preparation is as follows: to 100 ml. of an azoprotein solution containing 16.2 mg. of protein per ml. are added dropwise with stirring 6 ml. of an alum solution (100 mg./ml.) with the *p*H maintained between 7 and 8. The *p*H is finally adjusted to 7.4 and merthiolate added to a concentration of 1:10,000.

Preparation of Antisera.—Antisera were obtained from rabbits which had received 16 injections over a period of 4 weeks with increasing dosage of antigen. Each rabbit received a total of about 84 mg. of azoprotein. The rabbits were bled by cardiac puncture on the 4th, 6th and 8th days after their final injection and exsanguinated at the third bleeding. This procedure provided a maximum yield of optimum titer one-course serum, the total per rabbit ranging from 80 to 100 ml. The restriction to first-course sera was based on the consideration that the heterogeneity of the antibody might be reduced in view of the changes in the quality of antibody associated with prolonged immunization. The sera from 7 to 10 rabbits, from each group of 10 rabbits which had received the same antigen preparation over the same period of time, were pooled and used for antibody purification. The range of 7 to 10 represents the proportion of the rabbits whose sera contained antihapten antibody of the order of magnitude of 100 μg. N/ml. serum.

Purification of Antihapten Antibody.—A detailed description of the procedure we have developed for the purification of anti-I_p antibody will be published elsewhere. For our present purpose we may note several features of the procedure. The precipitating antigen is prepared from purified human fibrinogen (HF) by coupling the protein with the haptenic groups to give D-I_p–HF and L-I_p–HF. These azoproteins are further purified by precipitation with salt. The presence of anti-BγG antibodies in the antiserum is utilized to decomplement the serum by addition of BγG. The supernatant serum is then used for the specific precipitation of the anti-hapten antibody. The washed specific precipitate is extracted at *p*H 7.4 with the weakly bound hapten phenylacetate at a concentration of 0.1 *M*. This step serves to remove most of the antibody from the precipitate while only very little antigen is solubilized. The extracted antibody is precipitated three times with 1.6 *M* phosphate, *p*H 7.4. The use of K_2HPO_4 and KH_2PO_4 for this purpose avoids the introduction of nitrogen and also permits the maintenance of a constant *p*H. Our purification procedure is particularly mild since the entire process is conducted at *p*H 7.4 and, for the most part, at low temperature. It may be noted that a considerable concentration of the antibody is achieved, since about 50% of that present in the original pool of 600 to 1,000 ml. of serum is contained in 50 ml. of final solution.

The degree of specific precipitability of the purified antibody varies for different preparations. For some, such as P.A. 22 and P.A. 24, the antibody is completely precipitable except for the intrinsic solubility of the specific precipitate. In other cases, particularly where the yield is relatively low, complete precipitation of the antibody is not observed.

Binding Experiments.—Equilibrium dialysis was carried out with specially designed cells each of which consists of two identical sections. The sections were fabricated from Pyrex screw-top culture tubes. The tubes were cut near the top, the bottoms discarded and the cut ends of the upper portions flanged. The flanged ends were ground to provide

(9) D. Rudman, A. Meister and J. P. Greenstein, THIS JOURNAL, **74**, 551 (1952).

(10) A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **192**, 535 (1951).

(11) A. W. Ingersoll and R. Adams, THIS JOURNAL, **44**, 2930 (1922).

(12) Beilstein (2nd Supplement, XIV, p. 284) cites $[\alpha]^{15}D$ +84.1° in dilute KOH for benzoyl-[*d*(−)-α-aminophenylacetic acid].

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

a smooth surface with an inside diameter of 16 mm. and an outside diameter of about 23 mm. The cell is assembled in a brass frame with a washed cellulose disk (diam. 23 mm.) placed between the flanges. The cellulose disk divides the cell into two compartments and serves as the dialyzing membrane. The outside edges of the flanges are coated with paraffin wax to prevent leakage. The sections are closed with tinfoil-lined screw caps. The capacity of each compartment is about 1.5 ml.

The binding experiments were set up with 1 ml. of antibody solution in one section and 1 ml. of dye solution in the other section. The solutions were measured with calibrated pipets. The antibody usually was used at a concentration of approximately 750 μg. N/ml., corresponding to 3×10^{-5} M for a molecular weight of 156,000, in a solvent containing 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4. The dyes were also dissolved in the same solvent. Whenever an inhibitor was used it was initially contained in the dye solution. All binding determinations were done in duplicate.

Experiments were conducted at two temperatures, 25.0 and 7.1°. The cells were immersed in a constant temperature water-bath and rotated at a speed of 5 r.p.m. For the lower temperature a bath was used in a cold room. Control experiments, with albumin as the binding protein and with the dye initially mixed with the protein solution as well as with the dye initially in the non-protein compartment, showed that equilibration was attained after 24 hr. at 25.0° and 44 hr. at 7.1°. All the antibody experiments were done with these dialysis periods. It may be mentioned that the adequacy of our procedure has been demonstrated by the fact that the same results are obtained with albumin binding experiments when these are done on a 10-fold larger scale in the manner previously described.[13]

The free equilibrium dye concentration was determined by transferring the contents of the non-protein compartment to a 1-ml. absorption cell and measuring the optical density at λ470 mμ with a Beckman spectrophotometer using a path length of 5 or 10 mm. The range of free dye concentration encountered in this work was from 0.2×10^{-5} to 4×10^{-5} M. The molar extinction coefficient of the I_p dyes at λ470 mμ is 2.90×10^4. Calculation of the amounts of dye bound to the antibody required corrections for the quantity of dye bound to the cellulose membrane. For the D-I_p dye at 7.1° the correction was taken as 13% of the total quantity of free dye, while at 25° the amount adsorbed ranged from 8% to 14% depending on the free dye concentration. For the L-I_p dye at 25° a correction of 7% was used.

Absorption Spectra.—The absorption spectra of D-I_p dye in the presence of antibody were measured from λ600 mμ to λ320 mμ at room temperature. The concentration of the antibody was 2.48×10^{-5} M and two different total dye concentrations were used: 0.942×10^{-5} M (10 mm. path length) and 4.73×10^{-5} M (5 mm. path length). A binding curve at 25° was determined for the particular antibody preparation used above. From this curve it was calculated that at the lower dye concentration 93.0% of the dye was bound (r = 0.353, see below) and at the higher concentration 76.4% was found (r = 1.46). With these data the contribution of the free dye to the absorption could be taken into account and the absorption spectra of the bound dye calculated. A further small but significant correction due to the light scattering by the protein was also included.

Results and Discussion

The Binding of Azohapten by Purified Antibody.—The binding results for soluble complex formation between anti-D-I_p antibody and D-I_p dye are shown in Figs. 1 and 2 for two preparations of purified anti-D-I_p antibody. The curves are theoretical and are based on considerations presented below. The data are given in the form of a Scatchard[14] plot, r/c *vs.* r, in which r is the average number of dye molecules bound per antibody molecule at the equilibrium concentration of free dye c. In this kind of plot the extrapolation of the binding curve to the abscissa yields the binding capacity (valence) of the antibody. The devia-

(14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

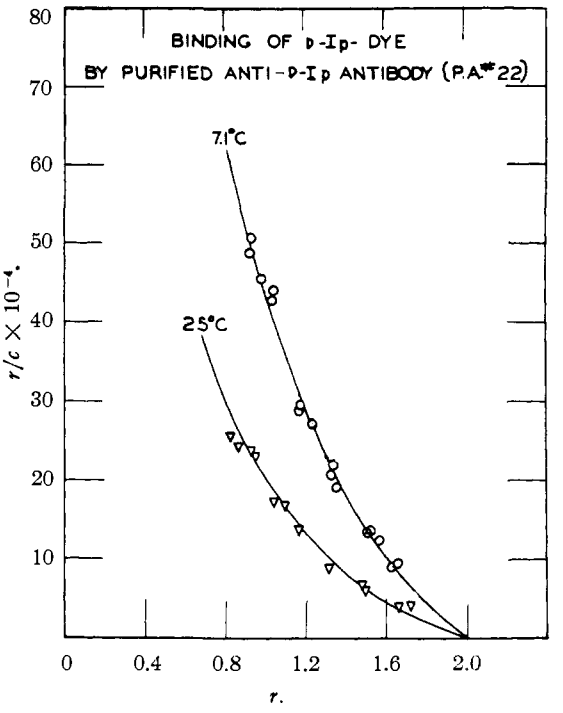


Fig. 1.—Binding results at 25 and 7.1° for the reaction between D-I_p dye and purified anti-D-I_p antibody (P.A. 22). The points are experimental and the curves are theoretical.

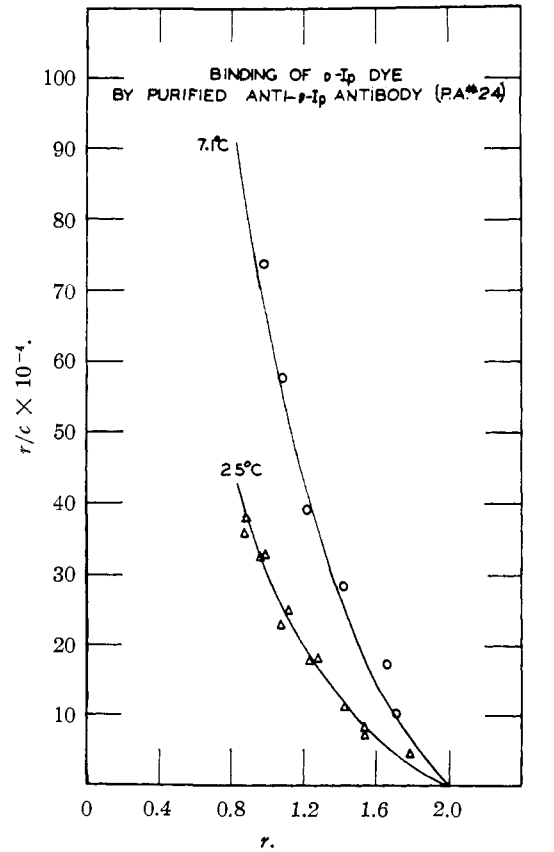


Fig. 2.—Binding results at 25 and 7.1° for the reaction between D-I_p dye and purified anti-D-I_p antibody (P.A. 24). The points are experimental and the curves are theoretical.

tion of the curve from linearity is a measure of the heterogeneity of the antibody with respect to its affinity for the dye. If all of the combining regions of the antibody possess the same intrinsic association constant, K, then the law of mass action leads to the relation

$$r/c = nK - rK \qquad (1)$$

where n is the antibody valence.

Inspection of the binding data shows that the observed values of r range from 0.8 to 1.8 with the consequence that a reliable extrapolation can be made. The value of 2.0 thus obtained for the valence of precipitating antibody is estimated to be accurate to within 5%. Since the experimental points do not fall on straight lines, it can be concluded that there exists a heterogeneity of the specific sites of the antibody. The average value of the association constant, K_0, for each curve is taken as the reciprocal of the free dye concentration at which $r = 1$, *i.e.*, at half-saturation of the antibody. Finally, it is apparent that the binding is temperature-dependent since the curves for 7.1° are considerably higher than the corresponding ones for 25°.

The specificity of the combination between antibody and the homologous azo hapten is readily demonstrated by the use of the isomeric azohapten. Thus, an anti-D-I_p antibody preparation with $K_0 = 3.50 \times 10^5$ for the D-I_p dye showed only slight binding of the L-I_p dye with an affinity constant approximately 1/35 as large as that for the homologous reaction.

Antibody Heterogeneity.—In order to provide a quantitative measure of the heterogeneity of the combining sites, we have derived theoretical binding curves in the manner similar to that used for albumin binding.[15] These are based on the assumption, first formulated by Pauling, *et al.*,[16] that the heterogeneity can be described by the Gauss error function in terms of the free energy of binding. On this basis the distribution of association constants is given by

$$\frac{dN}{N} = \frac{1}{\sqrt{\pi}} e^{-[\ln (K/K_0)]^2/\sigma^2} d[\ln (K/K_0)] \qquad (2)$$

where K_0 is an average binding constant and σ is the heterogeneity index. The fraction of sites occupied as a function of c is expressed by

$$r/n = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 c e^{\alpha\sigma}} d\alpha = 1 - f(c) \qquad (3)$$

in which α has been substituted for $\ln (K/K_0)/\sigma$. A value of 2 is assigned to n and the value of K_0 is easily obtained from the experimental binding curve by noting that $f(c) = 1/2$ for all values of σ when $K_0 c = 1$. The theoretical curves shown in Figs. 1 and 2 have been calculated with $\sigma = 2.3$. The numerical evaluation of the integral was carried out by the aid of the Gauss quadrature formula as described by Greenwood and Miller.[17] It is evident that the curves are in satisfactory agreement with the experimental results. Because of the limited range of data, however, it cannot be concluded that a Gaussian distribution in the free energy of combination, with $\sigma = 2.3$, constitutes a complete description of the heterogeneity of the purified antibody solutions.

Thermodynamic Results.—In Table I are summarized the results deduced from the binding data of Figs. 1 and 2. The value of 2.0 for the antibody valence has been obtained with six preparations of purified antibody. There is the possibility, of course, that 2.0 is an average value for a mixture of antibodies of different valences including, *e.g.*, 1 and 3. However, this appears most unlikely since the value 2.0 has been found consistently although the association constants at 25° for the six batches covered a range of 2×10^5 to 5×10^5.

TABLE I

THERMODYNAMIC RESULTS FOR THE BINDING OF D-I_p DYE BY PURIFIED ANTI-D-I_p ANTIBODY

			25°		71.°			
P.A.	n	σ	$K_0 \times 10^{-5}$	$-\Delta F°$, kcal./mole	$K_0 \times 10^{-5}$	$-\Delta F°$, kcal./mole	$-\Delta H°$, kcal./mole	$\Delta S°$, e.u./mole
22	2.0	2.3	2.05	7.25	4.4	7.24	7.1	0.3
24	2.0	2.3	3.1	7.50	6.7	7.48	7.3	.7

Our result for the valence of antibody is in agreement with that obtained earlier by similar procedures for another rabbit anti-hapten antibody by Eisen and Karush,[5] namely, anti-*p*-azobenzenearsonate. It is of considerable significance that the same result can be inferred for rabbit antibodies against protein antigens on the basis of electrophoretic analysis of solutions of antigen and antibody in antigen excess.[18,19]

It will be noted from Table I that the standard free energy for complex formation is between −7.2 and −7.5 kcal. per mole of hapten. For the group of six preparations which have been studied at 25° ΔF^0 ranges from −6.7 to −7.8 kcal. This range includes the value of −7.7 kcal. previously reported for the combination of *p*-(*p*-hydroxyphenylazo)-benzenearsonate with purified anti-*p*-azobenzenearsonate antibody at room temperature.[5] Doty and Epstein[20] have recently published a similar value for antibody with the same specificity. This was based on a study by light scattering of the reaction between rabbit antibody and two bivalent haptens, terephthalanilide-*p*,*p*′-diarsonic acid and adipanilide-*p*,*p*′-diarsonic acid.

Comparison with Albumin Binding.—Of particular interest is the fact that the specific binding is due almost entirely to the ΔH term, the entropy contribution being practically negligible. This result is in striking contrast to the binding behavior of serum albumins. These proteins bind anionic azo dyes very strongly, including the D-I_p and L-I_p dyes as we have shown for bovine serum albumin[13] and human serum albumin.[21] With the albumins, however, it is generally observed that the ΔH term is small or zero,[22] the major contribution to the

(15) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

(16) L. Pauling, D. Pressman and A. L. Grossberg, *ibid.*, **66**, 784 (1944).

(17) R. E. Greenwood and J. J. Miller, *Bull. Am. Math. Soc.*, **54**, 765 (1948).

(18) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3499, 4851 (1955).

(19) J. R. Marrack, H. Hoch and R. G. S. Johns, *Brit. J. Exp. Path.*, **32**, 212 (1951).

(20) P. Doty and S. I. Epstein, *Nature*, **174**, 89 (1954).

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

(22) I. M. Klotz, *Cold. Spr. Harb. Symposium Quant. Biol.*, **14**, 97 (1950).

free energy of combination coming from the large net increase in entropy. In Fig. 3 are shown binding curves for human serum albumin and D-I_p dye in 0.05 *M* phosphate, *p*H 7.0 at 25° and 7.1°. Although it is not possible to calculate a reliable value of ΔS^0, it is clear that the albumin binding is much less temperature-dependent than antibody binding. This is evident from a comparison of the ratios of the values of r/c at the two temperatures. Thus for the antibody this ratio is about 2, whereas for albumin the ratio ranges from about 1.3 to 1.0 depending on the value of r selected.

The difference in the contributions of the ΔS^0 terms for specific and non-specific binding suggests considerable structural dissimilarity of the combining regions of γ-globulin and serum albumin. In the case of the albumins we have previously concluded that there is a reversible structural alteration of the protein associated with the binding process, probably a slight separation of helical sections. The entropy increase invariably observed in the binding of anions is attributed, at least partly, to the rupture of intramolecular protein bonds. The necessity to break such bonds in albumin can account for and is, indeed, suggested by the observation that the ΔH^0 for the binding of D-I_p dye by albumin is evidently much less than that for anti-D-I_p antibody, although the affinities appear to be very similar. The ability of the combining regions of albumin to assume the variety of complementary configurations they must provide to account for binding, *i.e.*, their configurational adaptability, has been related to the probable existence of regions in the protein molecule in which the interhelical attraction is relatively weak.[21]

In the case of the antibody, on the other hand, the combining region probably consists of an interhelical cavity whose van der Waals contour is closely complementary to, and therefore selective for, the antigenic group. The contour of the cavity is determined by the relative positions of several amino acid side chains. The integrity of the cavity is maintained against the disruptive tendencies of thermal energy and of intramolecular electrostatic repulsion to which the molecule is subjected at extreme values of *p*H, *e.g.*, 3. It is to be expected, therefore, that the attractive interaction between the helical portions in the neighborhood of the combining region will be relatively strong in order to provide the necessary rigidity and stability.[23]

Combination of the hapten with the antibody involves a transfer of a largely hydrophobic molecule from an aqueous environment to a region of much lower dielectric constant. The number of hydrogen bonds which the free hapten can form with its neighboring water molecules is much less than that possible for an equal volume of water, which might be viewed hypothetically as occupying the hole remaining after binding of a hapten molecule. On the basis of this consideration complex formation would be exothermic and would be associated with a substantial decrease in the enthalpy of the system. The possible quantitative significance of this effect can be seen from an approximate calculation based

(23) We would suggest the possibility that intramolecular disulfide bonds exercise an important function in this connection.

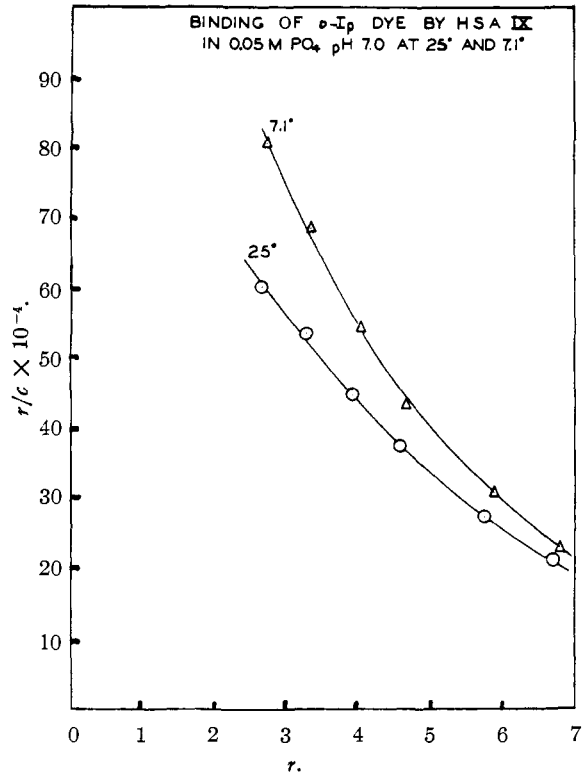


Fig. 3.—Binding curves at 25 and 7.1° for the reaction between D-I_p dye and human serum albumin.

on the value of 50 ergs per sq. cm. for the interfacial surface energy of an oil–water interface.[24] We consider the hapten to be equivalent to a cube with an area of 76 Å.^{2} per face. If $^1/_2$ of the total area consists of hydrophobic surface which, on binding to the protein, replaces an oil–water interface with an oil–oil interface, then we calculate that about 16 kcal. per mole of hapten should be released. If there is an unoccupied cavity in the antibody which is filled with water, its occupation by the hapten should provide additional stabilization of the same order of magnitude. In spite of the fact that the measured value of $-\Delta H$ is much smaller than 16 kcal., it is to be expected that enthalpy effects will constitute an important factor in the thermodynamics of antibody–hapten combination.

In the light of these considerations we may now suggest that in albumin binding the above enthalpy advantage is used for the separation of helical sections from each other accompanied by the rupture of interhelical linkages. Thermodynamically, therefore, the binding appears largely as a consequence of the entropic advantage acquired, although large energy effects may be involved. The occurrence of helical separation also serves to provide an explanation for some of the other features of albumin binding.[21]

Comparison with Protein–antiprotein System.—It is of interest to compare our results with those recently published by Singer and Campbell.[18] These authors studied the equilibrium formation of soluble antigen–antibody complexes by electro-

(24) *Cf.* H. Eyring, R. Lumry and J. D. Spikes in "The Mechanism of Enzyme Action," Eds. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 129.

phoresis and ultracentrifugation. The systems investigated consisted of protein antigens, bovine serum albumin and ovalbumin and their homologous rabbit antibodies. The thermodynamic results for the specific combination of antigen and antibody were very similar for the two systems within the unavoidably large experimental error. The results for the protein antigens differ from ours in that the intrinsic association constants are about 1/5 to 1/10 as large as those observed for the hapten–antibody combination. Furthermore, the reactions are characterized by a large positive contribution of ΔS^0 which appears to be the main driving force of the process. In contrast to this result we have found, as described above, that ΔS^0 is practically zero.

It is unlikely that these differences can be accounted for by the dissimilarities of the antigenic groups involved. For example, specific precipitates of protein antigen and antiprotein antibody do not exhibit a greater solubility than azoprotein–antihapten antibody precipitates. Quantitative precipitin curves also provide no evidence for a larger association constant for the latter system than for the protein–antiprotein reaction. The small value of the ΔH term in the results of Singer and Campbell is difficult to explain if it is taken to characterize the specific combination. Inasmuch as their association constants were measured at *p*H 8.5 ($\Gamma/2 = 0.3$), it might appear that electrostatic repulsion between the negatively charged antigen and antibody molecules would exercise a significant and non-specific effect on the constants. This possibility has been considered by these authors but, on the basis of the behavior of their systems as a function of *p*H, they have concluded that in the range of *p*H from 8.5 to 3.1 non-specific intermolecular electrostatic interactions do not significantly affect the antigen–antibody equilibrium.

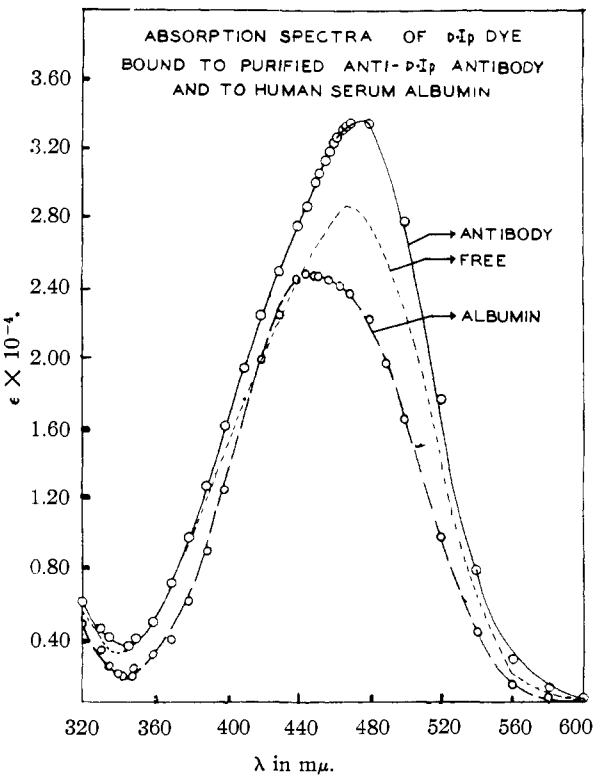


Fig. 4.—The absorption spectra of D-I_p dye free and combined with human serum albumin and anti-D-I_p antibody.

It appears to us, however, that electrostatic repulsion at *p*H 8.5 would be an important factor and that this factor could account for the differences in results noted above. Support for this view is found not only in the theoretical calculation of the electrostatic effect based on the theory of Verwey and Overbeek, made by Singer and Campbell[25] themselves, but also from the importance of the electrostatic factor inferred in other instances of protein association reactions, *e.g.*, the dimerization of α-chymotrypsin[26] and the association of insulin.[27] On this basis it can be argued that the enthalpy advantage associated with the specific interaction serves to compensate for the electrostatic repulsion. In addition an entropic gain may result from the decrease in solvent electrostriction accompanying complex formation. That is, the separation of charge which occurs on dissociation leads to a larger amount of bound water compared to that in the complex. Such an effect has been used to account for the entropy changes in the association of insulin.[27]

We may note also that in continuation of the studies of Singer and Campbell described above, Baker, *et al.*,[28] have investigated the reaction between antibody to the *p*-azobenzenearsonic acid group and benzenearsonic acid–*p*-azo-bovine serum albumin. Their results, at *p*H 8.5, $\Gamma/2$ 0.3 and 0°, yield a value of −3.8 kcal.[29] per mole of haptenic group for the association reaction. This is about one-half the value reported by Eisen and Karush[5] for antibody of the same specificity as well as by Doty and Epstein.[20] We believe that this large discrepancy provides additional evidence that electrostatic repulsion at *p*H 8.5 was important in the results discussed above.

Absorption Spectrum of Bound Dye.—The binding of the D-I_p dye to its homologous antibody results in a significant change in the absorption spectrum of the dye. This is shown in Fig. 4 which also includes the spectrum of the free dye for comparison. The same curve was obtained for two widely different values of *r*, 0.353 and 1.46. This result indicates that, with respect to the interaction responsible for the spectral shift, there is little variation among the antibody molecules. The shift consists of an increase in the molar extinction coefficient at the maximum from 2.90×10^4 to 3.36×10^4, an increase in the wave length of maximum absorption from λ470 mμ to λ475 mμ and a broadening of the absorption band.

The explanation for this spectral change cannot

(25) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3504 (1955).

(26) R. F. Steiner, *Arch. Biochem. Biophys.*, **53**, 457 (1954).

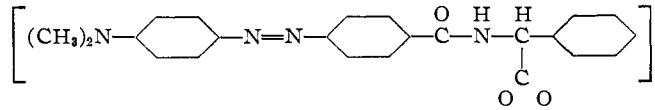
(27) P. Doty and G. E. Myers, *Disc. Faraday Soc.*, **13**, 51 (1953).

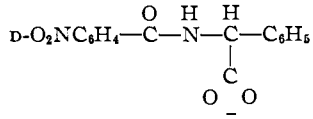
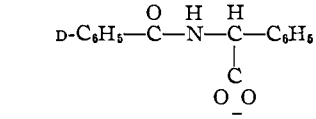
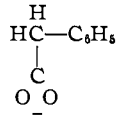
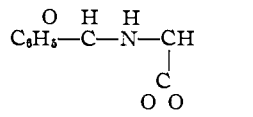
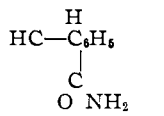
(28) M. C. Baker, D. H. Campbell, S. I. Epstein and S. J. Singer, THIS JOURNAL, **78**, 312 (1956).

(29) This value is obtained from their reported value of −4.8 kcal./mole for the reaction $Ag + AgAb \rightleftarrows (Ag)_2Ab$ by taking into account the statistical factor arising from the bivalence of antibody and multivalence (13.4) of the antigen.

TABLE II

THE INHIBITION OF DYE BINDING BY STRUCTURALLY RELATED MOLECULES AT 25°



	Anti·L·I_p antibody + L·I_p dye P.A. 29, K_0 = 49.5 × 10^4, $-\Delta F°$ = 7.77 kcal./mole		Anti·D·I_p antibody + D·I_p dye P.A. 30, K_0 = 29.4 × 10^4, $-\Delta F°$ = 7.48 kcal./mole		P.A. 33, K_0 = 8.5 × 10^4, $-\Delta F°$ = 6.74 kcal./mole	
	K_1 × 10^{-4}, l./mole	$-\Delta F°$, kcal./mole	K_1 × 10^{-4}, l./mole	$-\Delta F°$, kcal./mole	K_1 × 10^{-4}, l./mole	$-\Delta F°$, kcal./mole
	0.141	4.30	12.8	6.98	6.16	6.56
L-	15.6	7.08	0.0657	3.85	0.0371	3.51
	(2.69)[a]	(6.04)[a]	(8.77)[a]	(6.74)[a]	2.80	6.08
L-	(9.57)[a]	(6.80)[a]	(2.08)[a]	(5.90)[a]	1.86	5.84
	0.154	4.36	0.285	4.72	0.184	4.47
	.0100	2.73	0.00776	2.58	0.00554	2.38
	.011	2.8	(0.011)[b]	(2.8)[b]	.....	..

[a] These results were obtained with a preparation of the D-inhibitor which may have contained up to 8% of the L-form and a preparation of the L-inhibitor with a maximum of 14% of the D-compound. [b] These results were obtained with another anti-D-I_p preparation for which K_0 = 18.9 × 10^4.

be attributed to the fact that the bound dye is contained in a region of low dielectric constant. In organic solvents the absorption band of azo dyes usually shifts to lower wave lengths. In the case of the D-I_p dye in dioxane we have observed that the absorption maximum is lowered to λ430 mμ. A more likely interpretation is that a hydrogen bond is formed between a proton-donating group of the protein and the azo group of the dye. This interaction would lend stability to the resonance form of the dye in which the azo group carries a negative charge. Such stabilization would be expected to lead to spectral changes qualitatively consistent with those observed.

In the case of the binding of the D-I_p dye by serum albumins a spectral shift occurs which is entirely different from that described above. As may be seen in Fig. 4 this spectral shift consists in a decrease of λ_{max}, a decrease in ϵ at λ_{max} and a sharpening of the absorption band. We may account for the contrasting results with antibody and albumin on the same basis employed in our thermodynamic discussion. Whereas the homologous antibody provides a complementary cavity for the dye, the albumin molecule is subject to a rupture of interhelical bonds on combination with the dye. The resistance to such bond breakage would result in a small distortion of the planar *trans* configuration about the azo group so as to minimize the effect. The distortion of the planar structure would give rise to the qualitative changes found as may be seen from a comparison of the absorption spectra of the *cis* (non-planar) and *trans* forms of azo dyes.[30] Since azo dyes bound to albumin generally undergo the kind of spectral shift just described we may conclude that their interaction with these proteins is associated with some distortion of the planar *trans* form. This is the form which is otherwise the stable one in aqueous solution.

Inhibition Studies.—To obtain more detailed information about the complementary region of the antibody, we have studied the inhibition of dye binding by several structurally related molecules. For purposes of calculation it has been assumed that both the dye binding and the inhibitor binding can be described by single association constants. The association constant of the inhibitor, K_I, is obtained using the relation, derived from mass action considerations

$$K_1 = \frac{(r/r' - 1)(1 + K_0 c)}{(I)} \qquad (4)$$

where (I) is the equilibrium concentration of the inhibitor and r and r' are the number of molecules of

(30) W. R. Brode, J. H. Gould and G. M. Wyman, THIS JOURNAL, **74**, 4641 (1952).

dye bound per antibody molecule at the dye concentration c in the absence and presence of inhibitor, respectively. Since a significant amount of inhibitor may be bound to the antibody this quantity is calculated from the relation

$$r_1 = \frac{nK_1(I)}{1 + K_0c + K_1(I)} \qquad (5)$$

where r_I is the average number of inhibitor molecules bound per antibody molecule. On the basis of preliminary experiments the initial concentrations of the inhibitors were chosen so that, wherever possible, the amounts of dye and inhibitor bound to the antibody would be in the same range. This procedure serves to minimize the possible error introduced by the heterogeneity of the antibody in the determination of K_I. That is, the value of K_I calculated from equation 4 would then be very close to the average association constant which would be found if the binding of the inhibitor by the antibody were measured directly.

A summary of the inhibition results is presented in Table II showing the values of K_I for several inhibitors obtained with three preparations of purified antibody, two anti-D-I_p and one anti-L-I_p. There are also shown the calculated values of ΔF^0 at 25° for the combination of inhibitor with antibody. For comparison with these values there have been included the values of K_0 and ΔF^0 for the formation of the dye–antibody complex.

There are a number of interesting implications which may be drawn from the results of Table II. The homologous *p*-nitro derivatives are the most strongly bound inhibitors and exhibit a high degree of specificity. With respect to the anti-D-I_p antibody the D-inhibitor is bound about 200 times more firmly than the L-inhibitor. In the case of the anti-L-I_p antibody, on the other hand, K_I for the L-compound is more than 100-fold larger than the constant for the D-compound. Further, we note that the differences in the free energies of binding of the homologous dyes and the homologous *p*-nitro inhibitors are small, ranging from 0.2 to 0.7 kcal./mole. This result suggests that the size of the combining region of the antibody is probably not much larger, if at all, than that required to make contact with the entire dye molecule. It is interesting to note that Kabat[31] has recently reached a quite similar conclusion from inhibition studies with human antidextran antibody. He infers that the combining site is "complementary to an open chain of at least three α-D-glucopyranose units of homologous structure and probably to part or all of a fourth unit."

The D- and L-forms of the second inhibitor, phenyl-(benzoylamino)-acetate, show much greater cross-reactivity than that observed with the nitro compounds. The corresponding association constants may differ by a factor as small as 1.5 as shown by P.A. 33. With antibody preparations which bind the homologous dye more strongly, P.A. 29 and 30, the factor may be as large as four but apparently not much greater even if the possibility of optical contaminants is taken into account. This considerable cross-reactivity may arise from the ability of the phenyl group of the benzoyl substituent and the α-phenyl group to exchange their roles with respect to their location on the antibody in the case of the heterologous isomer. The addition of a *p*-nitro group, by virtue of its steric effect, would preclude such an exchange and account for the increased specificity of the nitro derivatives.

The contribution of the benzoylamino group to the interaction of the hapten with antibody is evident from the reduced inhibitory effect on its removal, as shown by phenylacetate. This group evidently contributes as much as 2.4 kcal. to the free energy of binding.

An even greater contribution to the binding is made by the α-phenyl group since the effect of its absence, in the form of hippuric acid, is to decrease the value of $-\Delta F^0$ by about 4 kcal. The quantitative superiority of the α-phenyl group over the benzoylamino group is probably due to the loss of hydrogen bonds with water molecules which the latter group must undergo on combination with antibody and which are not compensated for by the formation of new hydrogen bonds. We have also demonstrated the importance of the α-phenyl group in another way. Direct measurement of the binding of another dye which differs from the I_p dye only by the absence of the α-phenyl substituent has yielded an association constant which is at least 100-fold less than that for the homologous I_p dye.

Finally, a comparison of phenylacetate and phenylacetamide suggests that the anionic group also contributes substantially to the free energy of binding. We can only estimate a maximum value for this contribution, however, approximately 2 kcal., because the ability of the amide group to form hydrogen bonds with water molecules would tend to reduce the inhibitory effectiveness of phenylacetamide.

(31) E. A. Kabat, THIS JOURNAL, **76**, 3709 (1954).

PHILADELPHIA, PA.