1.27 g./ml. Calculated from this value, the hydration was 0.60 g. of water per g. of bacteriophage. Strictly, this value is the excess water in addition to solvent associated with 1 g. of dry bacteriophage in sucrose solution.[11] If the assumption is made that the sucrose solution does not change the bacteriophage in any way, then it is also the value for the water of hydration of the virus in water.

Since the calculated hydrations of the two components are identical, it is unlikely that the different sedimentation rates of the two components of T_2 bacteriophage are the result of a difference in hydration of the particles.

Shape of the Particles.—In principle, the sedimentation rate of a substance depends on the weight, hydration and shape of the particles. A change in any one of these properties might change the sedimentation rate. It appears that the two components of T_2 bacteriophage probably do not differ as a result of an aggregation or a change in hydration. If such is indeed the case, then the different sedimentation rates of the two components can be attributed to a change in shape.

The friction ratio, f/f_0, for a component can be calculated from the experimental friction coefficient f, and the friction coefficient for a spherical anhydrous particle of the same mass f_0, by means of the equations

$$f = RT/ND_{20}^{w} \quad (7)$$

$$f_0 = 6\pi\eta_w r_0 \quad (8)$$

$$r_0 = \sqrt[3]{3MV/4\pi N} \quad (9)$$

in which N is Avogadro's number, η_w is the viscosity of water at 20°, and r_0 is the radius of the spherical anhydrous particle of the same mass M. The friction ratios are 1.59 and 2.00 for the faster and slower sedimenting components, respectively.

The friction ratio is a function of the hydration and shape of the particles of material. The higher friction ratio for the slower sedimenting component indicates that this component (a) is the more hydrated, or (b) departs more from the spherical shape. Since evidence has been given that the hydrations of the two components are identical, the second alternative is the more likely. Thus, from all the evidence given above, the most likely deduction is that, when the *p*H of the medium is changed from *p*H 5 to 7, the bacteriophage particles change in shape in such a way as to result in a slower sedimentation rate, a smaller diffusion coefficient, and a higher friction ratio.

PITTSBURGH 13, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF INDUSTRIAL MEDICINE, NEW YORK UNIVERSITY POST-GRADUATE MEDICAL SCHOOL]

The Specific Interaction of Some Dinitrobenzenes with Rabbit Antibody to Dinitrophenyl–Bovine γ-Globulin[1]

BY MARY E. CARSTEN[2] AND HERMAN N. EISEN

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The specific binding of homologous haptens of different size to antibody has been studied at two temperatures by the method of equilibrium dialysis. The antibody was formed in rabbits against dinitrophenyl-bovine γ-globulin. Dinitroaniline and ε-dinitrophenyllysine were the haptens used in the binding studies. Relative binding affinities were obtained graphically. The hapten in which the determinant group was combined with amino acid in the same way as in the immunizing antigen, namely, ε-dinitrophenyllysine, was more strongly bound than the simpler hapten 2,4-dinitroaniline. Binding was somewhat higher at low than at high temperature. The antibody was found to exhibit considerable heterogeneity and a multiple number of binding sites. An antibody fraction which precipitated with the homologous antigen, showed greater homogeneity and a "valence" of approximately two.

The specific combination of antigen of high molecular weight with antibody has been studied extensively in the past. In most cases this combination leads to the formation of a precipitate. On the other hand, the specific interaction of antibody with certain homologous haptens of low molecular weight frequently results in the formation of soluble complexes. Because of their small size and solubility, these complexes are amenable to a physicochemical study from which information may be obtained concerning the number of binding sites (valence) of antibody, the free energy of binding of hapten and the specificity of the interaction of antibody with hapten.

In the present investigation the soluble complexes formed by rabbit antibody to 2,4-dinitrophenyl-bovine γ-globulin (DNP-bovine γ-globulin) with 2,4-dinitroaniline and ε-N-dinitrophenyllysine (ε-DNP-lysine) have been studied by the method of equilibrium dialysis. Marrack and Smith[3] were first to demonstrate by this method the specific binding of homologous hapten to antibody, after removal of serum albumin from the immune serum of rabbits immunized with an azoprotein. Haurowitz and Breinl[4] carried out a similar experiment without removing serum albumin, and instead compared the amount of hapten bound to immune serum with that bound to normal serum. In a quantitative study of purified antibody by this method, Eisen and Karush[5] determined the number of binding sites on an antibody and the free energy of binding of a hapten.

(1) (a) We wish to acknowledge the support of this work by grants from the Standard Oil Co. (N. J.), New York City, and the National Microbiological Institute of the National Institutes of Health, United States Public Health Service (Grant No. RG-1713). (b) Presented in part at a meeting of the American Association of Immunologists, *Federation Proc.*, **13**, 488 (1954).

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

(4) F. Haurowitz and F. Breinl, *Z. physiol. Chem.*, **214**, 111 (1933).

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

2,4-Dinitrobenzenes are of considerable interest as haptens. Some of these compounds react *in vivo* with tissue proteins,[6] and give rise to a variety of allergic phenomena which duplicate the biological responses to antigens of high molecular weight.[7] In addition, the non-ionic dinitrobenzenes afford an interesting contrast with the ionic haptens which have been widely studied.[8]

Our previous investigation of the interactions of dinitrobenzenes with serum albumin[9] has made it possible to compare specific with non-specific binding of these substances to proteins.

Experimental

Preparation of Antiserum.—Rabbits were immunized with bovine γ-globulin (Armour Laboratories, Fraction II) which had reacted with 2,4-dinitrobenzene sulfonate.[10] The antigen had an average of 8 DNP groups per γ-globulin molecule, as determined spectrophotometrically[11] and by micro-Kjeldahl analysis, respectively. For the spectrophotometric analysis an extinction coefficient of 17,400 at 360 mμ was used, *i.e.*, that of ε-DNP-lysine,[9] since dinitrobenzene sulfonate combines primarily with the free amino groups of the lysine side chains. The antigen was used in the form of an alum precipitate[12a] to which 0.3% phenol was added. The rabbits were injected intravenously four times a week for four weeks with about 1.5 mg. protein per injection. They were bled by heart puncture three days in succession, starting the fifth day after the final injection. The sera from each animal were combined and the best sera from 50 animals were pooled.

Purification of Antibody.—The globulin fraction of the antiserum was prepared by one precipitation with 1.75 *M* ammonium sulfate.[13] An electrophoretic analysis of this material showed it to be 63% γ-globulin, 18% β-globulin, 17% α-globulin and 2% albumin.[14] For use in the dialysis experiments phosphate buffer in 0.15 *M* sodium chloride was added to make the solution 0.05 *M* with respect to phosphate, *p*H 7.4. Protein concentrations were measured in a model DU Beckman spectrophotometer. The spectrophotometer reading was calibrated with micro-Kjeldahl nitrogen values for a solution of rabbit γ-globulin. The protein concentration was calculated, using a value of 16% nitrogen and a molecular weight of 160,000.[12b]

Assay of Antibody.—The amount of precipitating antibody in the γ-globulin fraction of the serum was estimated from quantitative precipitin curves.[11] The same DNP-bovine γ-globulin was used as antigen which had been used for immunization. From the amount of precipitated antibody, the amount of antibody precipitated with bovine γ-globulin was subtracted to obtain the precipitated anti-DNP antibody. This was considered valid because supernatants after antibody precipitation with bovine γ-globulin showed the same extent of binding with ε-DNP-lysine as the original rabbit γ-globulin solution. Furthermore, the amount of antibody precipitated with DNP-bovine γ-globulin was considerably diminished by previous treatment with bovine γ-globulin. Data on the precipitin curves of these systems are presented elsewhere.[11] Precipitated anti-DNP antibody was found to be 8.5% of the protein in the γ-globulin fraction.

From ε-DNP-lysine binding of the supernatants after precipitation with DNP-bovine γ-globulin it was evident that at no point of the precipitin curve had all the antibody been precipitated. An attempt was therefore made to estimate total antibody by adsorption on an insoluble antigen. Repeated treatment of the antigen (DNP-bovine γ-globulin) with dinitrobenzene sulfonate resulted in a preparation which had 29 DNP groups per bovine γ-globulin molecule. This antigen was soluble in water but insoluble in 0.15 *M* sodium chloride at *p*H 7. It was thus possible to utilize this preparation to adsorb antibody. The antigen was precipitated from water by adding sodium chloride to a concentration of 0.15 *M*. To the washed insoluble antigen the rabbit γ-globulin fraction was added. Antibody was completely removed from solution as ascertained by the absence of binding of a dilute solution of ε-DNP-lysine by supernatants of approximately 2% protein concentration. The insoluble material was washed twice with saline at 4°, then dissolved in 0.1 *N* NaOH and the amount of antibody was measured spectrophotometrically as before.[11] From the amount of antibody thus determined, antibody precipitated with bovine γ-globulin was again subtracted. This correction was required since the supernatants of the adsorption reaction no longer formed precipitates with bovine γ-globulin. By this procedure total anti-DNP antibody was found to be 9.8% of the protein. The non-precipitating antibody was only 13% of the total anti-DNP antibody. This antibody was found to account for 25% of the binding in a 2×10^{-5} *M* solution of ε-DNP-lysine.

Haptens.—Commercial samples of dinitroaniline were recrystallized twice. ε-DNP-lysine was prepared according to the method of Porter.[15] Concentrations of both haptens were estimated spectrophotometrically.[9] Over the range of concentrations studied, the hapten solutions obey Beer's law.

Dialysis Experiments.—Binding was measured by the method of equilibrium dialysis. All experiments were carried out in 0.15 *M* sodium chloride and 0.05 *M* phosphate buffer, *p*H 7.4 ± 0.05. The protein concentration was 2% and the initial concentration of hapten ranged from 2×10^{-5} to 40×10^{-5} *M*. To prevent bacterial growth, 200 μg. of streptomycin was added per ml. protein solution. The streptomycin did not modify binding of hapten.

Equilibration was carried out in specially designed glass tubes, consisting of two compartments, separated by a disc of Visking sausage casing. Each compartment was 18 mm. long, 16 mm. in diameter, had a screw cap at one end and a ground flange at the other. The disc of sausage casing was placed between the two flanges and the two compartments were held tightly together by threaded metal collars. One ml. of globulin solution was equilibrated with one ml. of a solution of hapten of varying concentration. Equilibration by gentle rocking (2 r.p.m.) in a constant temperature water-bath was complete after 24 hours at 25.0 ± 0.1° and after 48 hours at 5.0 ± 0.1°. All experiments were performed in duplicate. The amount of hapten bound was calculated from the concentration of free hapten at equilibrium and the initial concentration. Correction was made for adsorption of hapten to the cellophane. Control experiments were set up with the γ-globulin fraction from normal rabbit serum. These showed negligible (average 1%) binding of ε-DNP-lysine and only little binding of dinitroaniline, possibly caused by the albumin impurity. A correction in the calculation of dinitroaniline binding to antibody was made for non-specific binding. This correction amounted to 9% at 5° and to 4% at 25°.

The binding curve for precipitated antibody was obtained in the following manner. A binding curve of the rabbit γ-globulin fraction was set up after precipitation of the anti-DNP antibody with DNP-bovine γ-globulin. The binding values thus obtained were subtracted from those obtained with the total anti-DNP antibody at the same free hapten concentration. The difference is the binding due to precipitated anti-DNP antibody. From this and the known concentration of precipitated antibody in the γ-globulin fraction a binding curve for precipitated anti-DNP antibody was computed. Because of this method of calculation the over-all error is greater than for the other binding curves. Since some antigen stays in solution at the equivalence zone after precipitation of anti-DNP antibody,[11] binding with the antibody remaining in solution may have been inhibited to a small extent. Thus, the binding of precipitated antibody may be somewhat less than the data show.

(6) H. N. Eisen and S. Belman, *J. Exp. Med.*, **98**, 533 (1953).

(7) K. Landsteiner and M. W. Chase, *J. Exp. Med.*, **66**, 337 (1937).

(8) K. Landsteiner "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1945, p. 163.

(9) M. E. Carsten and H. N. Eisen, THIS JOURNAL, **75**, 4451 (1953).

(10) H. N. Eisen, S. Belman and M. E. Carsten, *ibid.*, **75**, 4583 (1953).

(11) H. N. Eisen, M. E. Carsten and S. Belman, *J. Immunol.*, **73**, 296 (1954).

(12) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C Thomas Publisher, Springfield, Ill., 1948, (a) p. 543; (b) p. 168; (c) p. 183.

(13) H. N. Eisen and D. Pressman, *J. Immunol.*, **64**, 487 (1950).

(14) We wish to thank Drs. Robert C. Warner and Ione Weber of the Department of Biochemistry, New York University College of Medicine, for carrying out the electrophoretic analysis.

(15) R. R. Porter in "Methods of Medical Research," The Year Book Publishers, Inc., Chicago, Ill., 1950, 3, p. 256.

Results and Discussion

The binding data are summarized in Tables I and II.

TABLE I

BINDING OF HAPTENS WITH RABBIT ANTI-DINITROPHENYL ANTIBODY IN 0.15 *M* NaCl, 0.05 *M* PHOSPHATE BUFFER, *p*H 7.4

Equil. concn. without globulin soln. $\times 10^5$ *M*	Equil. concn. (*c*) with globulin soln. $\times 10^5$ *M*	Concn. of bound hapten $\times 10^5$ *M*	Moles hapten bound/mole antibody *r*	*r*/*c* $\times 10^{-4}$
ε-Dinitrophenyllysine, initial antibody concn. = 1.14 $\times 10^{-5}$ *M*, 5°				
9.81	7.64	4.34	3.61[a]	4.72
7.58	5.89	3.38	2.97	5.04
6.62	5.12	3.00	2.63	5.14
5.70	4.17	3.06	2.69	6.44
4.75	3.42	2.66	2.21[a]	6.48
4.75	3.43	2.64	2.31	6.75
3.82	2.57	2.50	2.19	8.54
2.89	1.77	2.24	1.87[a]	10.5
1.94	0.99	1.90	1.66	16.8
1.52	0.643	1.76	1.48[b]	23.1
ε-Dinitrophenyllysine, initial antibody concn. = 1.20 $\times 10^{-5}$ *M*, 25°				
23.0	19.6	6.8	5.7	2.9
9.88	8.57	2.62	2.18	2.54
7.89	6.94	1.90	1.58	2.29
6.81	5.60	2.42	2.02	3.60
5.81	4.88	1.86	1.55	3.18
4.80	3.88	1.84	1.53	3.95
3.87	3.05	1.64	1.37	4.48
2.91	2.05	1.72	1.43	6.99
1.95	1.31	1.28	1.07	8.14
1.53	0.776	1.50	1.25	16.1
0.99	0.403	1.18	0.98	24.4
0.99	0.454	1.06	0.88	19.4
2,4-Dinitroaniline, initial antibody concn. = 1.20 $\times 10^{-5}$ *M*, 5°				
14.55[c]	13.55	2.00	1.66	1.22
7.35	6.59	1.52	1.26	1.92
5.91	5.18	1.46	1.22	2.35
4.48	3.77	1.42	1.18	3.14
3.04	2.35	1.38	1.15	4.90
2.33	1.65	1.36	1.13	6.88
1.61	0.986	1.24	1.03	10.5
1.31	0.728	1.16	0.97	13.3
2,4-Dinitroaniline, initial antibody concn. = 1.21 $\times 10^{-5}$ *M*, 25°				
15.98[c]	15.10	1.76	1.45	0.96
8.10	7.36	1.48	1.22	1.66
6.50	5.81	1.38	1.14	1.96
4.91	4.22	1.38	1.14	2.70
3.34	2.67	1.34	1.11	4.15
2.54	1.89	1.30	1.08	5.70
1.75	1.11	1.28	1.06	9.54
1.415	0.843	1.14	0.94	11.1
0.928	0.428	1.00	0.83	19.3

[a] Antibody concn. = 1.20 $\times 10^{-5}$ *M*. [b] Antibody concn. = 1.19 $\times 10^{-5}$ *M*. [c] This column gives the equilibrium concn. with the γ-globulin fraction from normal rabbit serum in order to correct for non-specific binding of 2,4-dinitroaniline (see text).

TABLE II

BINDING OF ε-DINITROPHENYLLYSINE WITH THAT RABBIT ANTIBODY WHICH IS PRECIPITABLE WITH DINITROPHENYL-BOVINE γ-GLOBULIN, IN 0.15 *M* NaCl, 0.05 *M* PHOSPHATE BUFFER, *p*H 7.4, AT 5°

Concn. of anti-dinitrophenyl antibody = 1.43 $\times 10^{-5}$ *M*. Concn. of precipitating anti-dinitrophenyl antibody = 1.24 $\times 10^{-5}$ *M*.

Equil. concn. without globulin soln. $\times 10^5$ *M*	Equil. concn. (*c*) with globulin soln. after precipit. with DNP-bov. γ-globulin $\times 10^5$ *M*	Concn. (*a*) of bound ε-DNP-lycine $\times 10^5$ *M*	Concn. (*b*) of bound ε-DNP-lysine in controls[a] not precipitated with DNP-bov. γ-globulin $\times 10^5$ *M*	Concn. (*b* − *a*) of ε-DNP-lysine bound to precipitating anti-body $\times 10^5$ *M*	Moles ε-DNP-lysine bound/mole precipitating anti-body *r*	*r*/*c* $\times 10^{-4}$
7.59	6.43	2.32	4.68	2.36	1.91	2.96
5.53	4.92	1.22	4.10	2.88	2.33	4.74
3.74	3.40	0.68	3.44	2.76	2.22	6.53
2.82	2.49	.66	3.06	2.40	1.94	7.78
1.86	1.72	.28	2.77	2.49	2.01	11.7
1.47	1.17	.60	2.49	1.89	1.52	13.0
0.948	0.724	.448	2.20	1.75	1.41	19.5

[a] These values were obtained by graphical interpolation of data given in Table I, corrected for antibody concentration of 1.43 $\times 10^{-5}$ *M*.

Figures 1 and 2 show the experimental results for the binding of the two haptens, ε-DNP-lysine and dinitroaniline, to antibody, plotted in terms of *r*/*c* *vs.* *r*. *r* is the average number of hapten molecules bound per antibody molecule at free hapten concentration *c*. Smooth curves were drawn through the experimental points and extrapolated to the vertical axis.

It is assumed that there are *n* binding sites per antibody molecule with intrinsic association constants K_i and that there is no interaction among bound hapten molecules. In this situation the relation between *r* and *c* is given by the law of mass action[16]

$$r = \sum_i \frac{K_i c}{1 + K_i c} \quad (i = 1,2,\ldots,n) \qquad (1)$$

From the law of mass action for the case of equal K_i's[17]

$$r/c = nK - rK \qquad (2)$$

The curve relating *r*/*c* to *r* will be a straight line unless the K_i's are not equal or there is electrostatic interaction between bound ions. As we are dealing here with substances of no net charge the latter possibility does not seem to be relevant. Furthermore, it has been found previously that electrostatic interaction did not account for non-linear binding curves obtained with ions.[16]

Even if the binding curves are not linear the intercept on the horizontal axis will equal *n*. Relative binding affinities can be obtained from the intercept on the vertical axis. As follows from equation 1[16]

$$\lim_{c \to 0} r/c = \sum_i K_i \qquad (3)$$

(16) F. Karush, THIS JOURNAL, **72**, 2705 (1950).
(17) G. Scatchard, *N. Y. Acad. Sci.*, **51**, 660 (1949).

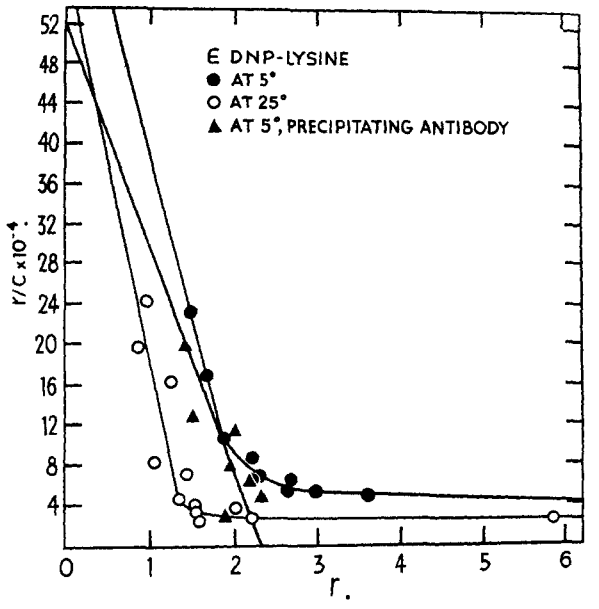

Fig. 1.—Binding of ϵ-dinitrophenyllysine with rabbit anti-dinitrophenyl antibody in a 2% γ-globulin solution, 0.15 *M* NaCl, 0.05 *M* phosphate buffer, *p*H 7.4.

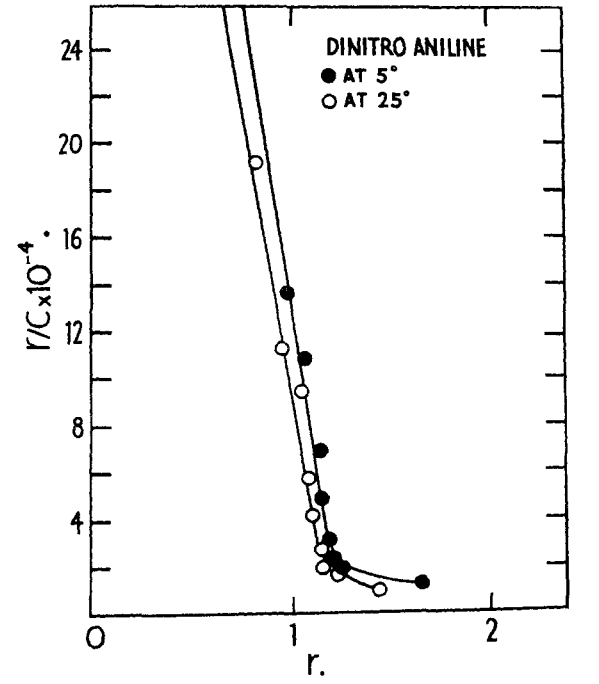

Fig. 2.—Binding of 2,4-dinitroaniline with rabbit anti-dinitrophenyl antibody in a 2% γ-globulin solution, 0.15 *M* NaCl, 0.05 *M* phosphate buffer, *p*H 7.4.

which is numerically equal to nK_0, where K_0 is an average intrinsic association constant. Because of the long extrapolation, these values may be of only limited quantitative significance, but may be used for purposes of comparison.

Binding Curves—Total Antibody.—The non-linearity of the binding curves may be ascribed to different values of K_i's, which means that the binding sites of antibodies are not equivalent. This interpretation is consistent with previous demonstrations of the heterogeneity of antibodies,[18,19] including those to single antigens.[20] In the present study, in which a derived protein was used for immunization, there probably exists a multiplicity of determinant groups as a result of substitution of different lysine side chains in bovine γ-globulin. Therefore, the possibility of obtaining antibodies with varying affinities for hapten would seem to be considerable. The binding curves indicate in particular that the first two binding sites are different from the others (Fig. 1), and that the first binding site may be stronger than the second (Fig. 2). The significance of the additional binding sites (Fig. 1) is not clear. That they represent specific binding seems probable because the control experiment with normal rabbit γ-globulin demonstrated that there was no significant binding in the absence of antibody. The presence of small amounts of serum albumin cannot account for these sites either, inasmuch as ϵ-DNP-lysine binding by serum albumin is negligible.[9] The antibody, however, was used in a mixture of proteins. Hence, it is conceivable that some unknown factors may have been responsible for these additional binding sites.

Because of the shape of the binding curves, extrapolation to the intercept on the *r*-axis involves a large uncertainty. Hence a value beyond doubt cannot be obtained for *n*. Nevertheless, the data may be interpreted to mean that *n*, the average "valence" of antibody toward hapten, is at least two, and is possibly four or more. These results are in accord with Heidelberger and Kendall's postulation of multivalence of antibody.[20] Plescia, Becker and Williams seem to have obtained a value above two for the "valence" of rabbit antibody, in contrast to horse antibody where a limiting value of two was found.[21]

Relative Binding Affinities.—As can be seen in Figs. 1 and 2, antibody has a greater binding affinity for ϵ-DNP-lysine than for dinitroaniline. Relative binding affinities are given in Table III. The values for $\sum_i K_i$, which is equal to nK_0, for the two haptens can be compared, assuming that *n* is the same for both haptens. The order of binding affinity is the reverse of that found with bovine

TABLE III

BINDING AFFINITIES AND BINDING ENERGIES FOR BINDING OF ONE MOLE OF HAPTEN BY HOMOLOGOUS ANTIBODY IN 0.15 *M* NaCl, 0.05 *M* PHOSPHATE BUFFER, *p*H 7.4

Antibody	Hapten	$\sum_i K_i \times 10^{-4}$ 5°	$\sum_i K_i \times 10^{-4}$ 25°	ΔH^0, kcal.	ΔF^0, kcal. 5°
Total anti-DNP	ϵ-DNP-lysine	70	57	−1.6	...
	Dinitroaniline	60	54	−0.9	...
Precipitable anti-DNP	ϵ-DNP-lysine	52[a]	..	...	−6.8

[a] $n = 2.3$. $K_0 = 2.3 \times 10^5$.

(18) K. Landsteiner and J. van der Sheer, *J. Exp. Med.*, **63**, 325 (1936).

(19) L. Pauling, D. Pressman and A. L. Grossberg, THIS JOURNAL, **66**, 784 (1944).

(20) M. Heidelberger and F. E. Kendall, *J. Exp. Med.*, **61**, 563 (1935); **62**, 697 (1935).

(21) O. J. Plescia, E. L. Becker and J. W. Williams, THIS JOURNAL, **74**, 1362 (1952).

serum albumin.[9] Indeed, binding of ε-DNP-lysine to bovine serum albumin was exceedingly small. The binding affinity of both haptens to specific antibody is strikingly larger than that to bovine serum albumin.

The greater binding affinity of the larger hapten, ε-DNP-lysine, to antibody is in accord with the view that van der Waals forces contribute considerably to the binding affinity of hapten for antibody although the possibility of ionic effects cannot be excluded in the case of the dipolar ion ε-DNP-lysine. Furthermore, from the relative binding of the two haptens it may be inferred that the antibody is formed not only as a complementary structure to the dinitrophenyl determinant, but also to the adjacent part of the protein molecule. A similar conclusion was drawn by Hooker and Boyd from a study of hapten inhibition of precipitation of antibody to azoproteins.[22] The ratio, however, of the intrinsic association constant of ε-DNP-lysine with antibody to that of dinitroaniline with antibody is close to one. This value is obtained by taking the ratio of the nK_0 values for the two compounds and assuming that n is the same for both. For this, three possible explanations may be offered: the number of binding sites of the antibody for dinitroaniline is larger than that for ε-DNP-lysine; or, the portion of the antigen to which the antibody is complementary is larger than ε-DNP-lysine; or, possibly, different antibodies are present. The participation in antibody formation of a portion of the antigen adjacent to the site of attachment of the hapten could account for the considerable heterogeneity of antibody. Each of the eight substituted lysine side chains of the antigen may be followed by different amino acids in the peptide chain. This difference in the immediate environment of the DNP-lysine residues in the antigen may produce a variety of determinant sites and thereby increase the heterogeneity of the antibody. As mentioned above, the presence of antibody sites of varying affinities is indicated by the shape of the binding curves. As a consequence of this heterogeneity, the information obtainable from a model system such as the present is limited.

Temperature Effect.—In Table III are given values for ΔH^0 for the binding of both haptens. It is assumed in their calculation that n is the same for the binding of each hapten at two different temperatures and that possible competitive effects of buffer ions are also the same. Both haptens show greater binding affinity at low than at high temperature, but the temperature effect is small.

Binding Curve—Antibody Precipitating with DNP-Bovine γ-Globulin.—As indicated above, most but not all of the anti-DNP antibody in the γ-globulin fraction can be precipitated by the immunizing antigen. The binding properties of this precipitable antibody are presented graphically in Fig. 1. The calculations are given in Table II. The binding curve was drawn as the best straight line, determined by the method of least squares. The shape of the curve indicates that the precipitated antibody has considerable homogeneity. The intercept of the straight line on the horizontal axis shows that n, the number of binding sites or "valence" of this antibody, is 2.3. This value agrees, within experimental error, with the "valence" of two obtained for purified antibody toward an azophenylarsonic acid hapten by the method of equilibrium dialysis.[5] It is also in agreement with the "valence" or precipitable antibody obtained by other procedures.[23]

Recently, a discordant view has been expressed by Banks, *et al.*,[24] who inferred a "valence" of one from their observation that an antigen–antibody precipitate, formed in the presence of homologous hapten, did not contain specifically co-precipitated hapten. They neglected, however, to take into account the fact that in the system of reversible equilibria only very small amounts of hapten could be bound to antibody in the precipitate, especially in the region of antigen excess, because antigen would be bound much more strongly than hapten. Furthermore, the method used would hardly reveal very small amounts of bound hapten. Their experiments, therefore, do not seem to furnish conclusive evidence regarding the "valence" of antibody.

The higher "valence" found for the antibody not precipitating with DNP-bovine γ-globulin than for the precipitating antibody need not be interpreted to mean that non-precipitable antibodies generally have a "valence" greater than two. The non-precipitating antibodies encountered in the present work may be an artifact, as it is known that antibody may change its ability to precipitate on partial purification.[12c]

The values for K_0, computed from the intercept on the vertical axis, and for ΔF^0 are given in Table III. The values for the association constant and ΔF^0 agree well with those obtained, also from binding data, for an azodye with homologous antibody.[5] The somewhat higher association constant and ΔF^0 observed in the latter work may be due to the following reasons: (1) the antibody used had been obtained by a purification procedure that may have yielded only a highly active antibody fraction, and (2) the hapten used was ionic, whereas dinitrophenyl used by us is non-ionic. Since ionic haptenic groups in antigens are believed to give rise to more highly specific antibodies than non-ionic groups,[8] it seems reasonable that the association constant should be lower for a non-ionic hapten, as found in this study.

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(22) S. B. Hooker and W. C. Boyd, *J. Immunol.*, **25**, 61 (1933).

(23) (a) A. M. Pappenheimer, Jr., H. P. Lundgren and J. W. Williams, *J. Exp. Med.*, **71**, 247 (1940); (b) L. Pauling, D. Pressman and D. H. Campbell, THIS JOURNAL, **66**, 330 (1944); (c) J. R. Marrack, H. Hoch and R. J. S. Johns, *Brit. J. Exp. Path.*, **32**, 212 (1951); (d) S. G. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952).

(24) T. E Banks, G. E. Francis, W. Mulligan and A. Wormall, *Biochem. J.*, **48**, 371 (1951).