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A Thermodynamic Study of Hapten–Antibody Association

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We have used the light scattering method and the theory of polycondensation equilibria to determine the thermodynamics of association in solution of a number of divalent haptens with pure preparations of rabbit anti-arsanilic antibody. The antibody molecules are linked together by the haptens containing two arsanilic acid groups, but the weight average degree of association rarely exceeds two. The association is reversible and the extent of association is clearly governed by an intrinsic equilibrium constant characteristic of the haptenic group and the antibody site. The average free energy of formation of the hapten–antibody bond is -7.4 ± 0.2 kcal./mole at antibody concentration of about 1 g./l. for the most strongly interacting haptens. This average value is lowered to -8.3 kcal. when the antibody concentration is reduced 10-fold, and this is interpreted as an indication of some inhomogeneity in the antibody sites. At a given antibody concentration the free energy values for the haptens investigated cover a range of about 1 kcal. and the variation is consistent with the expected contribution of steric hindrance. Measurements of the temperature dependence of the extent of association give $\Delta H^\circ = -0.8 \pm 2.6$ kcal./mole and $\Delta S^\circ = 22 \pm 9$ cal./mole/deg. These results indicate the liberation of a number of water molecules from the hapten and antibody molecules when the bond forms.

The antigen–antibody reaction including the formation of a specific precipitate appears to be the result of an aggregation of these molecules by a sequence of identical steps each of which is an association of a combining group on an antigen with a complementary site on an antibody to form an antigen–antibody "bond." This view has gained substantial support from the detailed formulation of Goldberg[2] because he was able to account for most of the observed features of these reactions on the assumption that the antigen–antibody bond was the product of a reversible association governed by a single equilibrium constant.

The direct observation of this equilibrium and the determination of its thermodynamic properties thus becomes a matter of obvious interest. However, typical antigen–antibody systems are unsuited for such a study because the multivalency of the antigen gives rise to reaction products that are too diverse and complicated to permit a determination of the extent of reaction. Moreover, the valency of the antigen, which is also required, is generally not known. These complications can be considerably minimized by lowering the extent of reaction to the point at which the number of reaction products become manageable. This course is being followed particularly by Singer and Campbell[3] who deduce the equilibrium composition from electrophoresis and ultracentrifuge patterns with the aid of suitable assumptions.

An alternative way of avoiding complex reaction products lies in replacing the multivalent antigen by a divalent hapten. If the divalency of the antibody is then accepted, the reaction products should be chains composed of alternating hapten and antibody. The extent of reaction could be determined by measuring the weight average molecular weight by light scattering and from this the equilibrium constant can be evaluated. The reversibility of the reaction should be readily demonstrated by noting the effects of adding one of the reactants to the already equilibrated mixture. Measurements at several temperatures should provide the data from which the heat and entropy of formation may be calculated. These expectations have been fulfilled as indicated in a preliminary note.[4]

(1) U. S. Public Health Pre-doctorate Fellow of the National Microbiological Institute of the National Institutes of Health (1952–53).

(2) R. J. Goldberg, THIS JOURNAL, **74**, 5715 (1952).

(3) (a) S. J. Singer and D. H. Campbell, *ibid.*, **75**, 5577 (1953); (b) **77**, 3499 (1955); (c) **77**, 3504 (1955); (d) **77**, 4851 (1955); (e) S. J. Singer, L. Eggman and D. H. Campbell, *ibid.*, **77**, 4855 (1955); (f) M. C. Baker, D. H. Campbell, S. I. Epstein and S. J. Singer, *ibid.*, **78**, 312 (1956).

Theoretical Considerations

With the assumption that all hapten–antibody bonds are equivalent, the equilibrium that is reached following the mixing of divalent hapten and divalent antibody is formally the same as that involved in the condensation polymerization of dibasic acids and diamines or diols analyzed by Flory[5] twenty years ago. The molecular weight distribution attained at equilibrium is dictated by the extent of reaction p, which we set equal to the fraction of haptenic groups reacted, and the ratio of haptenic groups to antibody sites, r. Because the weight of the divalent hapten is negligible compared to the antibody molecule, it can be neglected in considerations of the weight average molecular weight. As a consequence the quantity $pr^{1/2}$ of Flory's expression must be replaced by its square and the expression for weight average molecular weight, M_w, then becomes

$$\frac{M_W}{M_A} = \frac{1 + p^2r}{1 - p^2r} \qquad (1)$$

where M_A represents the molecular weight of the antibody. Since Goldberg's formulation[2] is a generalization of the simpler case treated by Flory, this same working equation can be obtained from his relations.

From the assumption of equivalence of the hapten–antibody bonds, it follows that if all of the molecules were cut in half, with one group or site per piece, the same number of bonds would form. Consequently the equilibrium constant with which we are concerned is that for the reaction

$$\text{hapten group} + \text{antibody site} \rightleftharpoons \text{hapten–antibody bond} \qquad (2)$$

that is

$$K_i = \frac{(\text{hapten–antibody bonds})}{(\text{free haptenic groups})(\text{free antibody sites})} \qquad (3)$$

Since r is known experimentally, p can be deter-

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

(5) P. J. Flory, THIS JOURNAL, **58**, 1877 (1936).

mined from the measurement of M_w and the use of eq. 1. The equilibrium constant, K_i, can then be evaluated as follows.

If H_0 and A_0 represent, respectively, the initial concentration of haptenic groups and antibody sites (twice the molecule concentrations), the product pH_0 is, equivalently, the concentration of bonds or of reacted haptenic groups or antibody sites, since each reacted haptenic group requires the reaction of one site and results in one bond. Hence

$$K_i = \frac{pH_0}{(H_0 - pH_0)(A_0 - pH_0)} = \frac{p}{(1 - p)(A_0 - pH_0)} \quad (4)$$

The standard free energy of formation of the bonds is

$$\Delta F^\circ = -RT \ln K_i \quad (5)$$

and measurements at several temperatures enable the standard heat and entropy of formation to be evaluated.

$$\frac{d \ln K_i}{dT} = \frac{\Delta H^\circ}{RT^2} \quad (6)$$

$$\frac{\Delta H^\circ - \Delta F^\circ}{T} = \Delta S^\circ \quad (7)$$

The light scattering method offers the only practical means of evaluating M_w and hence the equilibrium constant. This method provides the weight average molecular weight explicitly; its use does not disturb the equilibrium; it is sufficiently sensitive that only reasonable amounts of antibody are required for the observation.

The aggregate weight, M_w, is derived from the well-known light scattering equation applicable to transverse scattering[6]

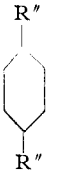
$$\frac{Kc}{R_{90}} = \frac{1}{M_w} + 2Bc \quad (8)$$

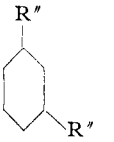
Since the aggregate weight is dependent on the concentration, the usual method of implementing this equation by extrapolating Kc/R_{90} to zero concentration cannot be applied. Instead we aim to work at single concentrations each of which is so low that the term $2Bc$ is negligible. Measurements described below on the pure antibody show that in the concentration range employed here (less than 0.1%) B is zero within probable experimental error. The aggregate sizes observed are sufficiently small for us to expect the same conclusion to apply to them. This conclusion is consistent with observations on most proteins at the ionic strength employed. Hence, we have taken M_w as equal to the reciprocal of Kc/R_{90} for the hapten–antibody mixtures.

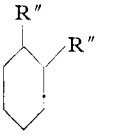
Materials and Experimental Methods

Haptens.—Six divalent haptens containing the phenylarsonic acid group were employed in the study of hapten–antibody equilibria. In addition a trivalent hapten was used in the preparation of the antibody. The names, structural formulas and code names of these haptens are listed below.

(6) See, for example, P. Doty and J. T. Edsall, *Adv. in Protein Chemistry*, **6**, 35 (1951). The low dissymmetries observed permitted the neglect of angular dependence of scattering.

Terephthalanilide-*p,p'*-diarsonic acid  T

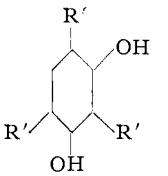
Metaphthalanilide-*p,p'*-diarsonic acid  Meta-T

Orthophthalanilide-*p,p'*-diarsonic acid  Ortho-T

Oxanilide-*p,p'*-diarsonic acid R″—R″ O

Carbanilide-*p,p'*-diarsonic acid R″—NH—C$_6$H$_4$—AsO$_3$H$_2$ C

Adipanilide-*p,p'*-diarsonic acid R″—(CH$_2$)$_4$—R″ A

1,3-Dihydroxy-2,4,6-tri-(*p*-(*p*-azophenylazo)-phenylarsonic acid)-benzene Often designated "R$_3$'-Resorcinol"  D

R′ denotes –N=N–C$_6$H$_4$–N=N–C$_6$H$_4$–AsO$_3$H$_2$ and R″ denotes –CO–NH–C$_6$H$_4$–AsO$_3$H$_2$.

Antisera.—Horse serum proteins were coupled with diazotized arsanilic acid as described by Boyd,[7] employing one gram of arsanilic acid for each gram of protein nitrogen. After coupling, the arsanilic–protein compounds were purified by repeated precipitation with acid, washing and resolution in water by the addition of minimal amounts of NaOH. The final *p*H was 8.1. For injection a 1% solution of this material in 0.15 *M* NaCl was prepared and a 10% suspension of killed streptococci added as an adjuvant, in the proportion of 50 ml. of the bacterial suspension to 70 ml. of arsanilic–protein solution.

Rabbits were injected three times a week with this mixture for four weeks[7] and bled from the marginal vein of the ear after a one week rest. After three bleedings the rabbits were given another three injections and bled again. This schedule was followed, replacing animals which died, until about 5 liters of serum had been obtained. On the basis of precipitability with *D*, these sera contained 0.5 to 1.0 mg./ml. of anti-arsanilic (anti-R) antibody.

Antibody Preparations.—Two distinct methods were employed in preparing purified antibody.

In both procedures we first precipitated the γ-globulin fraction three times at about one-third saturation with ammonium sulfate. A portion of an approximately 2% solution of the fraction in *p*H 8 buffer was analyzed with *D* for anti-R, that is the antibody directed against the azophenylarsonic acid group. Antibody was then precipitated at the optimal proportion. When the precipitate had stood 24 to 60 hr., it was centrifuged off, washed and purified by one of the procedures below. The antibody was obtained as a 0.1 to 0.5% solution.

Method A. Example, Purification of Pool 53-7.—This procedure was similar to the method described by Campbell, *et al.*[8] A precipitate containing 150 mg. of anti-R and 1400 μg. of D was dissolved by gentle stirring in 50 ml. of *p*H 3.1 HCl solution, 0.15 *M* in NaCl. The trivalent hapten D is insoluble at this *p*H but is apparently colloidally dispersed in the presence of anti-R. However, when the solution was centrifuged at 40,000 r.p.m. (Spinco Model L ultracentri-

(7) W. C. Boyd, "Fundamentals of Immunology," 2nd Ed., Interscience Publishers, New York, N. Y., 1947.

(8) D. H. Campbell, *et al.*, THIS JOURNAL, **70**, 2496 (1948).

fuge, rotor 40) for successive periods of three, two and two hours, precipitates were deposited each time, which had a D-anti-R mole ratio[9] of about 6 and contained a total of 550 μg. of D but only 11 mg. of anti-R. Since the remainder of D was concentrated toward the bottom of the tubes, the final supernate was divided into three fractions according to color. Each was dialyzed against *p*H 8 buffer; thereupon all but a trace of the remaining D precipitated, together with part of the anti-R, leaving a total of 70 mg. protein, a yield of 45%. Two other pools, 53-9 and 53-11, were also purified in this way.

Method B. Example, Purification of Pool 54-2.—The observation that the barium salt of D was insoluble at *p*H 8 suggested the following method. A specific precipitate containing 800 mg. of anti-R was dissolved in 80 ml. of 10% sodium arsanilate soln. at *p*H 8.3, and 8 ml. of saturated $BaCl_2$ solution was added. But the barium salt formed a colloidal suspension; it was necessary to centrifuge (35,000 r.p.m., rotor 40) for about 7 hr. before most of the D was deposited. When the supernatant solutions were dialyzed against *p*H 8 buffer, almost all the remaining D precipitated, leaving 520 mg. protein in solution, a yield of 65%. We noted that if the protein concentration of the dissociated precipitate exceeded 1%, the D sedimented too slowly to be removed effectively.[10]

Traces of D were present in these purified solutions; those with a concentration of 0.5% protein were noticeably tinted. We found by colorimetry on one preparation (pool 53-11) that about one molecule of D was present per 15 antibody molecules.

During these experiments the solutions were stored as long as two months at 5° in *p*H 8 buffer. A small amount of sediment usually developed during this time, but there was no evidence of any progressive loss of activity or other signs of denaturation. Thus the extent of aggregation of hapten T with freshly prepared pool 53-11 was identical with that found after storage of the pool for 4 months.

The salts employed were analytical reagent grade. The *p*H 8.0, $\mu = 0.15$ buffer was prepared by titrating 0.2 *M* H_3BO_3, 0.15 *M* in NaCl, with 0.15 *M* NaOH.

Light Scattering Details.—The photometer used was the Brice–Speiser instrument, with a removable 3 mm.-wide slit system. Fluorescence was shown to be absent in hapten–antibody mixtures at the wave length used, 436 mμ. The following paragraphs describe our modifications of usual procedure.[11]

Two kinds of cells were used. When the dissymmetry (using angles 45 and 135 deg.) or the scattering envelope was desired, R_{90} was measured in "partitioned cells" (Pyrocell Co., 207 E. 84th St., N. Y., N. Y.). These permit angular measurements on about 5 ml. of solution which occupies the rectangular section to which the incident beam is restricted so that only solvent is required for the semicylindrical section. The intensity of radiation at 30 to 135 deg. from very dilute fluorescein solutions, when multiplied by the sine of the angle is constant (±1 to 4%). The partition cell was removed to obtain the incident intensity.

Other measurements were made with 1 cm. square silica cells. To reproducibly position these, we adapted a sawed-off quarter of an ordinary Beckman Spectrophotometer 4-cell holder and screwed it to a plate which fit the table of the photometer. A platform was inserted in the holder so that the beam just missed the cell bottom and we could then make measurements on 1.5 ml. of solution.

The partitioned cells had a "cell constant" which ranged from 1.20 to 1.35 (±0.02) and the Beckman cells from 1.30 to 1.41, depending presumably on the lamp in use. In addition, the molecular weight of bovine serum albumin determined using the 1-cm. cells was found to be 67,000 ± 2000, within 2% of the values obtained in a square cell and a dissymmetry cell for the same sample.

Temperature dependence measurements were made in a partitioned cell with a round bottom, which was inserted into the thermostated metal holder in use in this Laboratory.[11]

All solutions were centrifuged in a Spinco Model L Ultracentrifuge for 1 hr. at 36,000 or 40,000 r.p.m. (in rotor 40). The usual aluminum caps were coated thinly with "Glyptal" varnish (Gen. Electric Co.). For clarifying the smaller volumes required when Beckman cells were used, tubes were made by shaping a polyethylene rod like a standard tube and drilling it out to a wall thickness of $^1/_{16}$ in. and to such a depth that the capped tube had a 5-ml. capacity. A short piece of polyethylene rod served as cap; it was machined to fit tightly into the tube to prevent leakage and tube collapse but had a $^5/_8$ in. diameter shoulder to keep it from being driven down the tube.

Pipets and cells were rinsed with optically clean redistilled water after preliminary soaking in either detergent solution or sodium dichromate–sulfuric acid mixture and rinsing with ordinary tap and distilled water. Solutions were examined in a strong beam of white light at low angles to detect dust. Those we studied were judged to contain insignificant amounts. The dissymmetries of the solutions studied in the partitioned cells provide a more objective criterion for dust contamination.

Separate dilutions of antibody were made up and centrifuged. We abandoned the usual technique of adding increments of a concentrated solution to solvent because the required stirring denatures some protein. For the same reason, hapten and antibody were first mixed and then centrifuged. Unless otherwise noted, each mixture was individually made up volumetrically, using the several stock antibody preparations, whose concentration had been determined by optical density, and hapten solutions made up by weight.

We used the value 0.200 for the dn/dc of antibody, obtained on pool 53-11, using a Zeiss Rayleigh–Haber–Löwe type interferometer. We ascribe an uncertainty of 5% to our value. The other figure we might reasonably have used, that for human γ-globulin,[12,13] adjusted to 436 mμ using the Cauchy formula, is 0.196[14] and would cause no important change in our conclusions. For optical constant K we used $6.45 \cdot 10^{-7}$.

Determination of Protein Concentration.—The concentration of protein in the antibody preparations was determined spectrophotometrically at 279 or 280 mμ using $E^{1\%}_{1\,cm}$, as 15.0 in *p*H 8 buffer.[9] The absorption of solutions in the silica cells was measured directly. The contribution of hapten to the absorption in mixtures was taken as that of a solution of hapten of identical concentration, since the absorption of hapten and antibody in mixtures proved additive at 280 mμ, within probable error. We found values of 0.038, 0.057, 0.060, 0.081, 0.048 and 0.006 for the optical density per μg./ml. of *ortho-T*, *meta-T*, *T*, *C*, *O* and *A* haptens, respectively, at 280 mμ and in *p*H 8 buffer.

There is some uncertainty introduced in the concentration of hapten and antibody in the clarified solutions because some ultraviolet absorbing material was lost during cleaning in the ultracentrifuge. This is illustrated in Table I.

TABLE I

LOSS OF ULTRAVIOLET ABSORBING MATERIAL ON CENTRIFUGATION OF HAPTEN-ANTIBODY MIXTURES, COMPARED WITH A BLANK

Materials: Pool 53-4 (described below) antibody, hapten *T*, *p*H 8 buffer; centrifugation: one hour. 40,000 r.p.m., Spinco rotor 40, standard tubes.

Initial r	Optical density (at 280 mμ) Before centrifugation	Optical density (at 280 mμ) After centrifugation
4.25	1.082	1.008
0.89	0.769	0.674
0.18	.712	.650
0	.695	.656

In calculating our results we subtracted a part of the initial hapten and antibody absorption (known from the amounts put in the mixture), using the assumption that the

(9) D. Gitlin, *J. Immunology*, **82**, 437 (1949).

(10) In the case of pool 53-4, instead of ultracentrifuging, the hapten-dissociated precipitate was mixed with some of the barium borate formed by mixing solutions containing equal moles of $BaCl_2 \cdot 2H_2O$ and $Na_2B_4O_7 \cdot 10H_2O$. The D is preferentially adsorbed, leaving mostly anti-R in the supernate. The yield was only about 30%, perhaps because the borate also adsorbed much protein.

(11) H. Boedtker and P. Doty, *J. Phys. Chem.*, **58**, 968 (1954).

(12) G. E. Perlmann and L. Longsworth, THIS JOURNAL, **70**, 2719 (1948).

(13) S. H. Armstrong, Jr., *et al.*, *ibid.*, **69**, 1747 (1947).

(14) Campbell, *et al.*, ref. 8, found $dn/dc = 0.178$ (adjusted to 436 mμ), which is actually characteristic of β-lipo-proteins, which are 75% lipid. See ref. 13).

"lost" material had $r = 1$ (equimolar hapten and antibody). This procedure appears reasonable because we used for scattering the portion of the tube close to the rotor center, from which the heavier aggregates, which approach a composition of $r = 1$, might have partially sedimented. Alternatively if the antibodies sediment individually, they still may carry along 1 or 2 haptens per antibody molecule. Our assumption leads us to calculate a drop in r if the mixtures are initially in antibody excess and a rise in mixtures initially containing excess hapten.

Experimental Results

Antibody Molecular Weight and Existence of Equilibrium Association.—We record first some measurements on the antibody itself and other evidence that justifies in part our adoption of the model system described in the introduction.

Our first consideration is the absolute value of the molecular weight of the antibody and its uniformity in different preparations. The molecular weight of each antibody sample, determined by light scattering, is recorded in Table II and some of the data are plotted in Fig. 1.

TABLE II

MOLECULAR WEIGHTS OF ANTIBODY PREPARATIONS

Solvent: *p*H 8.0 buffer.

Pool designation	M_w
53-4	158,000 ± 10000
53-7	168,000 ± 10000
53-9	162,000 ± 7000
53-11	163,000 ± 11000
53-12	192,000 ± 15000
54-1[a]	159,000 ± 12000
54-2[a]	160,000 ± 5000
54-3[a]	164,000 ± 5000

[a] Pools 54-1, 2 and 3 were later mixed and the total pool designated 54.

Each pool except 53-12 has, within probable error, the reported molecular weight of rabbit antibody, 160,000.[15,16] This shows that the antibody is unaggregated and the assignment of a valence of two is thereby justified. Moreover in Fig. 1 we have the evidence that B is essentially zero for antibody solutions as previously indicated.

If we turn next to the association itself the first point requiring proof is the reversibility of the reaction. A mixture of T and anti-R in *p*H 8 buffer having an antibody concentration of 0.1 g./100 ml. and a r value of 1.0 exhibited a value of 3.25×10^{-6} for Kc/R_{90}. To one part of this mixture hapten T was added to bring the r-value up to 30: at equilibrium Kc/R_{90} was equal to 5.4×10^{-6}. To another part of the mixture a monovalent hapten, sodium arsanilate (in *p*H 8 buffer) was added to raise the r-value to 95: Kc/R_{90} rose to 6.0×10^{-6}. These

(15) See Ch. 6, E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C Thomas, Publisher, Springfield, Illinois, 1948. Campbell, *et al.*, also obtained 160,000 by light-scattering for anti-R, but they used a value of dn/dc that must now be considered too low. The error so incurred may have been cancelled by a complementary error in the value taken for the turbidity of CS_2, used as a reference standard.

(16) A year later one of us, S. I. E., found at Yale University that about 85% of pool 54 sedimented as one component with the constant of γ-globulin. Some faster-sedimenting material, probably an antibody dimer, was present. We have disregarded the effect of these aggregates on the association, because they may have occurred only in pool 54 and because of the uncertainty as to how many sites are active on such aggregates.

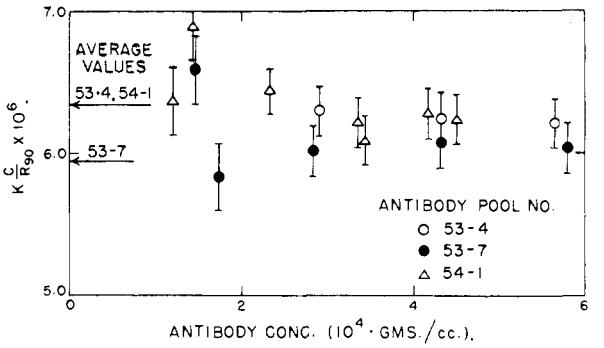


Fig. 1.—Light scattering determination of the molecular weight of various antibody preparations in *p*H 8 buffer.

experiments show that the aggregate weight of 310,000 in the original mixture was lowered by an excess of either divalent or monovalent hapten to 185,000 and 167,000, respectively. These values are within less than 10% of the values anticipated on the basis of equation 1 or its equivalent for monovalent hapten. Experiments of this type together with those described subsequently establish the reversibility within the limits of error of our measurements.

Constant Temperature Results.—The study of the equilibria themselves consisted of light scattering observations on mixtures of at least one of the haptens with each of the eight antibody preparations at several values of r. These results are collected in Table III where each row represents a light scattering experiment. The experimental data are listed as M_w/M_A which is equal to $R_{90}/160{,}000Kc$. The antibody concentration was determined by correcting the initial concentration by an amount computed from the loss in optical density as previously described. The hapten concentration is similarly corrected and the value of r is based upon these corrected concentrations. From these data we have computed the values of p, K_i and $\Delta F°$. Since the dissymmetries, z, were about the same as those found for solutions of antibody alone (1.01–1.05), we have made no correction to R_{90}. The measurements were taken at 23 ± 2°.

Upon examining Table III one notes that the greatest aggregation occurs in nearly equimolar mixtures and that, in general, it decreases in the presence of an excess of either reactant. The extent of association is relatively small in all cases, the average not exceeding that of an antibody dimer. By using the distribution functions of Flory[5] or Goldberg,[2] one can compute that in a typical case (antibody concentration = 4.3×10^{-4} g./cc., $r = 0.95$ and $K_i = 3.5 \times 10^5$) 58% of the antibody exists as monomer with zero, one or two attached haptens, 28% exists as the antibody dimer with one, two or three attached haptens, 10% exists as the antibody trimer with two, three or four attached haptens, 4% exists in the tetramer form and 1% in the form of still higher polymers. With only one average available we cannot, of course, check this distribution.

Free Energy Values.—From the results in Table III one notes that the values of the free energy of formation of the hapten–antibody bond

TABLE III

LIGHT SCATTERING FROM HAPTEN-ANTIBODY MIXTURES

	Protein concn. 10^4, g./cc.	r	$\frac{M_W}{M_A}$	z	p	10^{-5} K; (l./mole)	$-\Delta F^\circ$ (kcal./mole bonds)
Pool 53-4 and	3.8	4.8	1.48	1.04	0.20	15	8.42
Hapten T	4.1	0.86	1.58	1.04	.51	3.7	7.59
	4.1	0.10	1.13	1.04	.77	6.9	7.96
	4.0	4.8	1.51	1.04	.205	35	8.92
	4.0	0.87	1.58	1.05	.51	3.7	7.59
	4.3	0.10	1.12	1.04	.73	5.5	7.83
Pool 53-4 and	3.9	0.93	1.50	1.04	.46	3.1	7.49
Hapten A	3.7	5.3	1.34	1.08	.17	3.3	7.53
	4.0	26	1.12	1.03			
Pool 53-7 and	3.7[a]	5.6	1.71	1.05			
Hapten T	4.1	0.91	1.59	1.05	.50	3.6	7.58
	4.6	0.11	1.06	1.06	.62	3.0	7.47
Pool 53-9 and	3.6[a]	5.5	1.47	1.03			
Hapten T	3.6[a]	0.92	1.53	1.03	.48	3.6	7.58
	4.4	0.12	1.03	1.04	.34	1.0	6.82
Pool 53-11 and	8.7[b]	1.33	2.02	1.13	.50	2.9	7.46
Hapten T	8.5	1.01	1.92	1.07	.56	2.8	7.43
	9.0	1.01	1.92	1.05	.56	2.6	7.38
	9.0	1.01	1.90	1.05	.55	2.5	7.36
	9.0	1.01	1.90	1.05	.55	2.5	7.36
Pool 53-12 and	8.0	0.97	2.14	1.08	.61	3.9	7.62
Hapten T	8.1	5.2	1.85	1.06			
Pool 54-1 and	8.2[c]	0.98	1.64	1.04	.50	1.9	7.20
Hapten T	8.5[c]	5.0	1.45	1.04	.19	5.0	7.77
Pool 54-2 and	8.4	0.98	1.74	1.05	.53	2.2	7.29
Hapten T	8.3	5.3	1.42	1.03	.18	4.7	7.74
	9.2	0.99	1.75	1.06	.53	2.0	7.23
	9.3	5.3	1.42	1.03	.18	4.8	7.75
Pool 54-3 and	7.7	0.97	1.75	1.05	.53	2.5	7.36
Hapten T	7.4	5.3	1.46	1.03			

[a] Some precipitation occurred in these mixtures originally, greatly increasing the turbidity and dissymmetry. The measurements were on the recentrifuged solutions, which were stable. [b] This mixture made up after centrifugation of the components separately. [c] Precipitation developed in these mixtures. Initial turbidities were used in calculation in conjunction with optical density after precipitation since it appeared that the amount of precipitate was negligibly small.

are about -7.7 ± 0.6 kcal. Actually the reliability of the data varies considerably because of the increased sensitivity of the computed value of K to the measured ratio M_W/M_A as r departs from the value of unity. If only the more reliable values are averaged, the result obtained is -7.4 ± 0.2 kcal. at an antibody concentration of 0.08 g./100 cc. The principal use of the data taken for cases where r is far removed from unity lies in showing that within the probable experimental error the same equilibrium constant applies over a wide range of relative concentration. The extreme situation occurs in mixtures with r equal to about 5: in this case some values of M_W/M_A slightly exceed the permitted maximum, $(1 + 1/r)/(1 - 1/r)$, although they are within a few per cent. of values consistent with the value of ΔF° obtained for the mixtures of equimolar hapten and antibody concentration.

In general neither the value of R_{90} nor the dissymmetry of these solutions changed during the 2-day period after mixing and centrifuging in which observations were made. Consequently the results describe a stable equilibrium. Indeed, in one case the angular envelope (R_{30} to R_{135}) was found to be unchanged after one week at room temperature. However, in a few cases strongly scattering floccules formed within a few hours after clarification. This was preceded by increases in dissymmetry and scattering. Nevertheless, when the mixtures were reclarified, the ultraviolet absorption had dropped a total of only about 20% indicating that the amount of precipitate was relatively small. Further, the scattering from the reclarified mixtures was now stable; consequently, we have included these measurements, with the usual assumption as to the composition of the lost solute. Whatever the cause of the precipitation, the scattering from the soluble portion is essentially the same as that in mixtures which stay clear.

Table III also contains a measurement of the scattering from a solution of directly mixed hapten and anti-R, stirred by gently rocking the cell. The scattering measured 10 minutes after mixing remained constant for 24 hr. If we neglect the dissymmetry, caused by some dust, the turbidity is within error that of a similar pre-mixed solution. Since the equilibrium is rapidly reached, most of the measurements described below were made soon after the solution was taken from the centrifuge.

The Dependence of the Extent of Association on Hapten–Antibody Ratio.—The data of Table III suggest that aggregation is maximal in hapten-antibody mixtures at some optimum r-value. For hapten T and pool 53-11 this dependence of aggregation on r was determined in more detail

and such measurements permit us to test whether the form of the dependence of aggregation on r is that expected for the model system we have used to analyze the data. The observations are shown in Fig. 2, in which Kc/R_{90} for 13 mixtures with the

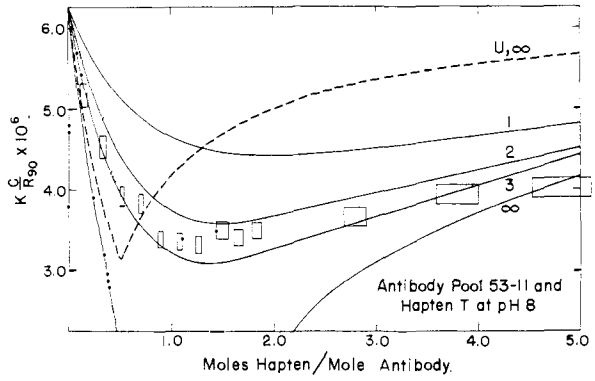


Fig. 2.—The dependence of the light scattering on hapten–antibody ratio. The antibody concentration is held constant at 0.043 g./100 ml. The full curves are drawn for the indicated values of K_iA_0, as described in the text. The dashed curve represents the minimum possible value of Kc/R_{90}, and hence the maximal aggregation, for mixtures of univalent antibody and divalent hapten.

same antibody concentration but different r-values is plotted against r. The effect of the uncertainty as to the composition of the mixture is also illustrated; the extreme values of r indicated are the initial value in the unclarified solution and that calculated as already described.[17] The theoretical curves were drawn for comparison in the following way. From equation 4 we obtain

$$K_iA_0 = \frac{p}{(1-p)(1-pr)} \qquad (9)$$

If we now choose various values of K_iA_0 and hence, for a given antibody concentration, various values of K_i, we can then use (9) to obtain p and from equation 1, M_w/M_A at each r-value.[18] Taking M_A = 160,000, we then have M_w and hence Kc/R_{90}. We find that over much of the range of r-values considered, the data lie along one such theoretical curve and thereby support the model here employed.[19]

We include in the graph a plot (U, ∞), indicating the maximum aggregation that a divalent hapten could bring about were we to assume that the antibody is univalent.[2] The aggregation found exceeds this maximum in hapten excess and is thus inconsistent with univalence of the antibody. This result confirms that found by the equilibrium dialysis method.[20,21]

The Dependence of Aggregation on Concentration.—The above results were obtained at only two levels of antibody concentration. For antibody whose sites are identical as we have supposed, the observations of Kc/R_{90} over a range of antibody concentrations for mixtures of one hapten–antibody ratio should fit a curve for a particular value of K_i calculated using equations 4 and 1. Data of this kind were obtained for the case of r = 1 and are plotted in Fig. 3. It is apparent that they are

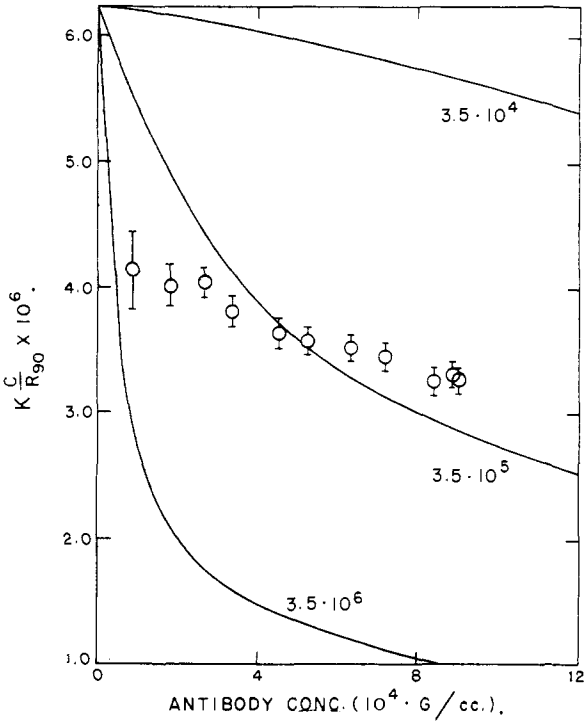


Fig. 3.—The concentration dependence of light scattering for hapten–antibody mixtures with the same r-value; equimolar hapten T and Pool 53-11 mixtures in pH 8 buffer. The curves are calculated for the indicated values of the intrinsic association constant K_i and r = 1, as described in the text.

not well-fitted by a single value of K_i. The value of K_i calculated from the highest concentration data is (2.6 ± 0.4) 10^5, substantially lower than that calculated from the the lowest concentration data, (11 ± 3) 10^5 Thus our simple picture of the association appears only partially valid. A reasonable interpretation of this inadequacy is that the sites are heterogeneous and that the K_i we obtain is an average value, but this view must be tentative since no other decisive indication of heterogeneity was found here. It is of interest to note that the highest antibody concentration used in our work is comparable to that occurring in high titer sera.

The Influence of Hapten Structure on the Association.—The union of a hapten molecule

(17) When the initial value of r is less than 1, the maximum average number of bound haptens per antibody molecule is also less than 1, so that in these cases, it might be true that the centrifuged solution retains the initial composition.

(18) Note that the curves so obtained have a minimum at $r = (K_iA_0 + 1)/K_iA_0$. The r-value at which aggregation is maximal thus depends on both K_i and A_0. That an optimal proportion for aggregation exists in this system reflects the ability of excess of either reactant to inhibit the aggregation.

(19) The value of K_i may be obtained at once from these data by dividing the number designating the curve on which they lie by the antibody site concentration in these mixtures. Since the antibody concentration in these mixtures was 0.043%, A_0 is 5.4 × 10^{-6} mole/liter. In addition it is found that K_iA_0 lies between 2 and 3, and consequently we obtain an estimate of K_i of between 3.7 and 5.6 × 10^5 in agreement with the results found for the same pool in the independent measurements shown in Table III.

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

(21) L. S. Lerman, Thesis, California Institute of Technology, 1949

with two antibody molecules brings the latter into such close proximity that steric effects on the free energy of bond formation may well be expected. In order to examine this possibility quantitatively, the scattering as a function of antibody concentration at r-values of unity was measured for five haptens. The concentration range covered was about tenfold; each dilution was centrifuged and analyzed spectrophotometrically. The results are plotted in Fig. 4. The results for hapten T coincide

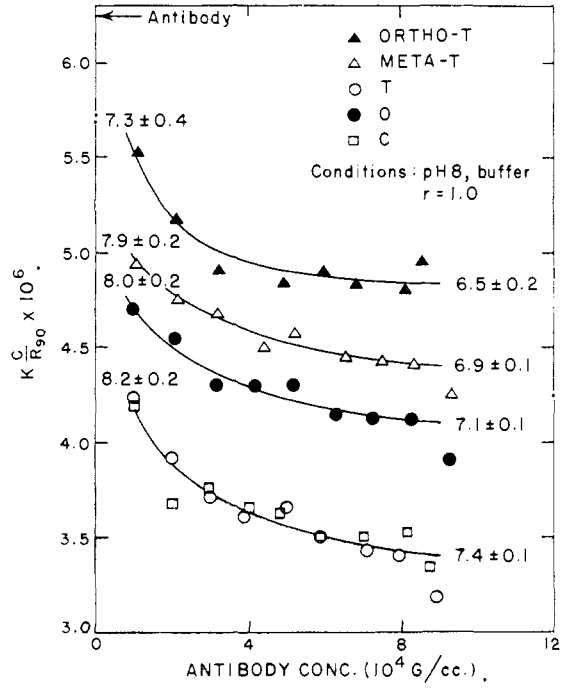


Fig. 4.—Comparison of light scattering from mixtures of five different haptens with the same antibody pool 54. The numbers at the end of each set of data are the values of $-\Delta F^\circ$ and the associated experimental error, calculated for the highest and lowest concentrations examined. Errata in the legend: the open triangles represent data for C and not meta-T; the open squares represent data for meta-T.

with the results given in Fig. 3 for a different antibody preparation. The results for the five haptens are seen to spread out in such a way that the values of the free energy of formation at any concentration cover a range of about 1 kcal. In each case there is a dependence of ΔF° on the antibody concentration, but this variation does not obscure the real differences in ΔF° among the different haptens.

The Temperature Dependence of the Association.—The value of the free energy of hapten-antibody bond formation only indicates the position of equilibrium. As in other studies of protein association,[22] additional insight into the formation of the bond may come from measurements of the heat and entropy of bond formation which can be derived from measurements of the equilibrium constant at different temperatures. These measurements have been made with hapten T in pH 8 buffer and are summarized in Table IV.

It is seen that the value of Kc/R_{90} rises about 10% in the temperature range of 15 to 37°. The values listed are reproduced if the solution is carried again through the same temperature cycle. The small change is indicative of a small value of the heat of bond formation and as a consequence some care must be given to the temperature dependence of K and c. Exclusive of the change in the specific refractive increment, dn/dc, this change in temperature would affect Kc by less than 1%. Instead of measuring the temperature dependence of dn/dc itself, the temperature dependence of the scattering of the antibody solution alone was determined and found to increase 5% over this range. As a consequence the observed values of Kc/R_{90} were multiplied by 1.03 at 15° and divided by 1.02 at 37° before being used to compute the values of M_w/M_A and K_i listed in Table IV. In this way the heat of bond formation was found to be -0.8 ± 2.6 kcal./mole.

If this is combined with the value of 7.4 kcal./mole for $-\Delta F^\circ$, the entropy of bond formation is found to be 22 ± 9 entropy units.

Discussion

For the purposes of comment the foregoing results may be summarized as follows. (1) Highly purified rabbit antibody preparations have been made by two methods and have been found to have a molecular weight of 160,000. (2) Antibody molecules are linked together upon the addition of divalent hapten and the dependence of the extent of association of the haptenic group–antibody site ratio, r, as well as the reversibility of the association demonstrates that the extent of association is governed by an intrinsic equilibrium constant characteristic of the interaction of the haptenic group and antibody site. (3) The average free energy of formation of this bond is -7.4 ± 0.2 kcal. (4) Variation of the antibody concentration over a 10-fold range shows a 10% variation in the free energy of hapten–antibody bond formation which is interpreted as a result of some degree of non-uniformity among the antibody sites. (5) There is a variation of a similar magnitude when structurally different divalent haptens are employed at constant antibody concentration. (6) Variation of the extent of the reaction with temperature leads to values of -0.8 kcal. for the heat and 22 e.u. for the entropy of formation of the hapten–antibody bond.

The two methods employed for purifying the antibody led to preparations having the same molecular weight (160,000) and the same reactivity with a given hapten not only at a given concentration but over a 10-fold range of antibody concentration. This latter observation shows that the average free energy of association is the same in the two preparations and in addition that the small variation with concentration, which we ascribe to inhomogeneity of antibody sites, is also the same. It is, therefore, a particularly sensitive indication of the reproducibility as well as the purity of these preparations.

The assumption of completely active antibody preparations is implicit in the foregoing interpretation of our data and is to be expected from the mildness of the procedures used in the isolation. This assumption was given additional support by an electrophoretic analysis of a mixture of arsanilic

(22) P. Doty and G. E. Myers, *Disc. Faraday Soc.*, **13**, 51 (1952).

TABLE IV

TEMPERATURE DEPENDENCE OF THE LIGHT SCATTERING FROM T–ANTI-R MIXTURES

r	(Pool 54) Antibody concn. 10^3, %	T, °C. → 15	$10^6(Kc/R_{90})_T$ 25	37	10^{-5} K_i 15	37	ΔH° (kcal./mole bonds)
1.0	9.6	3.36	3.42	3.66	2.09	1.91	−750 ± 2650
1.0	9.5	3.43	3.51	3.75	2.00	1.80	−900 ± 2600
5.2	9.7	4.18	4.30	4.42			
1.4	10.0	3.33	3.45	3.56	3.94	3.65	−650 ± 3950
0	9.6	6.34	6.49	6.75			
0	16.0	5.98		6.30			

$\Delta S^\circ = 22 \pm 9$ e.u./mole bonds at 25

acid coupled bovine serum albumin (RBSA) and antibody of pool 54 carried out by Professor S. J. Singer. This analysis showed the presence of the RBSA, present in excess, and complexes but no component with the mobility of γ-globulin. This demonstration of the absence of detectable amounts of inactive antibody is particularly welcome because it eliminates the possibility that the observed concentration dependence of the equilibrium constant could have been due to the presence of inactive antibody. (Only if about two-thirds of the antibody preparation were inactive could the data in Fig. 3 be fitted with a single equilibrium constant.)

In connection with this evidence of essentially completely active antibody, it is of interest to record that the maximum amount of antibody precipitated from these preparations by the trivalent hapten D ranged from 60 to 75%. It is our belief that the remaining antibody was involved in small soluble aggregates. It seems likely that the steric hindrances that come into play when three antibody molecules are bound to a single hapten substantially lower the free energy of bond formation and that as a result the extent of reaction is not carried far enough to yield a complete precipitate. Consequently this incompleteness of precipitation is not taken to indicate a corresponding lack of activity of part of the antibody preparation.

At this juncture some comparison between the precipitability of our antibody preparations and those of Pauling and co-workers[23] and Pardee and Pauling[24] is relevant. In the earlier work[23] it was found that a different divalent hapten than any employed here could bring about a precipitation of as much as 29% of the antibody from antiarsanilic sera. When in the latter work[24] purified antibody was employed it was found that 33 to 49% precipitated at antibody concentrations (0.034 g./100 cc.) near ours when their divalent hapten (chromatropic acid-R'_2) was used. This amount of precipitate occurred at $r = 1$; somewhat more was found at $r = 2$ and as much as 15% was found at $r = 0.25$ and 66.

In contrast we found no precipitates with our six divalent haptens except for small amounts (about 10% of the antibody) in about half of the antibody preparations: following removal of this precipitation the preparations were thereafter non-precipitating with divalent hapten. Clearly if we had encountered the behavior found by Pardee and Pauling this study could not have been undertaken.

(23) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda and M. Ikawa, THIS JOURNAL, **64**, 2994 (1942).

(24) A. B. Pardee and L. Pauling, *ibid.*, **71**, 143 (1949).

Pauling, *et al.*,[23] offer two possible explanations of the extensive precipitation they observed with divalent haptens: " . . . either the long strings themselves precipitate easily, or . . . enough trivalent antibody molecules are present to link the strings together." Since it is found in this study that the chain aggregates are both *soluble* and quite *short*, the first possibility is excluded. The second, which requires trivalent antibodies, remains a possible explanation but we think it more likely that the required polyvalent (greater than divalent) antibody units are aggregates of antibody molecules, perhaps only dimers, that exist because of slight denaturation or substantial difference in molecular charge. (The distribution of isoelectric points within preparations of γ-globulin is well known.) Once these polyvalent antibody units have been removed, the behavior of the remaining antibody can only be explained in terms of divalency. Consequently, we conclude that the precipitation in the Pardee–Pauling antibody preparation was due either to a much greater amount of aggregated antibody than we encountered or to association of their hapten or to both.

We turn now to the comparison of the values of the free energy of hapten–antibody formation with values for related associations. The most relevant comparison is with the value obtained by Eisen and Karush[20] and Lerman[21] by equilibrium dialysis studies of monovalent hapten binding. Their results are essentially identical with what we get at comparable concentrations for the T and meta-T haptens. If the equality of the free energy values in these two cases is accepted, then it follows that no steric hindrance occurs when these divalent haptens unite two antibody molecules over that which exists when a single antibody molecule combines with a haptenic group. The equivalence of these bonds was, of course, assumed in our analysis.

A comparison with other free energy values is made in Table V from which it is seen that the value for this particular hapten–antibody system is about as high as any yet observed in associating systems of this kind and is substantially higher than that now found for the serum albumin–antiserum albumin system or for the RBSA-anti-R system. Thus it appears that this particular choice of haptenic group and its location in a small hapten molecule rather than on another protein molecule creates the conditions for the most intense display of the specific forces involved in this type of biological activity. Yet this type of association can be weakened as much as 3 kcal. in free energy (or a factor of 150 in the equilibrium constant) and still exhibit the

TABLE V
THERMODYNAMIC STUDIES OF ANTIGEN-ANTIBODY AND OTHER MACROMOLECULAR ASSOCIATIONS

	$-\Delta F^\circ$ (kcal./mole)	$-\Delta H^\circ$	ΔS° (cal./mole/deg.)
Arsanilic haptens–rabbit antibody			
(1) Monovalent hapten (H Acid-R)[21]	6.8 (39°)		
(2) *p*-(*p*-Hydroxyphenylazo)-phenylarsonic acid[20]	7.7		
(3) T (this work)	7.4 ± 0.2	0.8 ± 2.6	22 ± 9
(4) Other divalent haptens (this work)	6.3–8.3		
ε-Dinitrophenyllysine–anti-DNP antibody[25]	6.8	1.6	17
Bovine serum albumin–anti-bovine serum albumin[3a,b]	5.5 ± 0.2	0 ± 2	20 ± 8
Ovalbumin–anti-ovalbumin[3d]	5.6 ± 0.2	0 ± 2	20 ± 8
R-Bovine serum albumin ("RBSA")–anti-R[3f]	5.2 ± 0.2	0 ± 2	18 ± 8
Insulin self-association[23]	5.2	7.7	−9

phenomenon of specific precipitation in protein–antiprotein systems because of the "gel-point" phenomena[2] that come into existence when the antigen valency exceeds two.

The values of ΔF^0 obtained by Singer and coworkers[3] bear further comment. Not only is their 2 kcal. value for the RBSA-anti-R system more positive than ours, but since their values refer to the reaction

$$\text{antigen} + \text{antigen–antibody} = (\text{antigen})_2\text{–antibody} \quad (10)$$

their value of the free energy of this reaction differs from that for the intrinsic association (see Goldberg[2]) by a statistical factor of $RT \ln (13/2) = 1.1$ kcal., since the number of haptenic groups per RBSA molecule was 13. Hence the actual difference is a matter of 3 kcal. in the free energy. The possibility that the antibody preparations were the source of most of this difference has been eliminated by the duplication of these results by Professor Singer using our antibody pool 54. Thus, in the absence of error in the interpretation of these results, it appears possible that orientation of the haptenic groups on the surface of the protein is such as to decrease substantially the associating tendency over that displayed in the simple hapten–antibody system.

The inhomogeneity of antibody sites found here is not new although the possibility of characterizing their distribution by this method has special merit. The range we estimate in our studies is similar to that which may be inferred from the literal interpretation of the published results of Eisen and Karush.[20,26]

The variations in the free energy values for the different haptens were not as great as might have been expected. Thus the shortening of the distance between the haptenic groups as occurs in O and C relative to T did not greatly diminish their efficiency at linking antibody molecules together. Moreover, it is only when the two groups are in neighboring positions on the central benzene ring that the effectiveness drops off. (Compare T and meta-T with ortho-T.) A more concrete comparison of these variations is afforded by listing the values of p^2 calculated from the scattering at a given antibody concentration. This quantity is equal to the fraction of hapten molecules which link two antibody molecules, that is, approximately the fraction of antibody dimers. For the haptens T, meta-T, O, C and ortho-T, respectively, this is 0.26, 0.26, 0.18, 0.15 and 0.12 at an antibody concentration of 0.04 g./100 cc.

These considerations suggest that if the antibody site is a cavity in the antibody surface, it is a shallow cavity, since the unreacted group of a bound hapten remains nearly equally accessible even though the distance between the two arsanilic acid groups is reduced to approximately 5 Å. as when T is replaced by O.

We turn finally to the heat and entropy data. Other relevant data are collected in Table V for comparison.

We see at once that the heat liberated when the hapten–antibody bond forms is negligibly small, of the order of thermal energy (kT). The same appears to be true for the antigen–antibody systems currently studied by Singer,[3] and the results of studies of certain protein associations such as insulin[23] also show a quite small heat of reaction. In all these cases the driving force of the reaction cannot be the gain in energy, or heat content, that accompanies such an association. The only reasonable interpretation seems to be that given originally for the case of insulin association.[23] In all of these cases one would expect the loss in entropy upon association to be quite large because of the decrease in translational and rotational freedom which these large molecules undergo. However, the measured entropy change is quite small. In this particular case it is calculated to be about −100 e.u. but the measured value is +22 e.u. The net entropy gain rather than a large loss in entropy upon association seems only explicable if bound solvent has been released upon formation of the hapten–antibody bond. Since 5 cal./mole/deg. is the entropy gain of water upon the analogous melting of ice, we must postulate something of the order of 24 water molecules being displaced from the haptenic group and antibody site when the bond is formed and entropy gain of these released water molecules then accounts for most of the driving force of the association. The possibility that some of this entropy gain resides in configurational changes in the antibody structure cannot be ignored, but this cannot in any event account for the major part of the total entropy gain.

(25) M. E. Garsten and H. N. Eisen, THIS JOURNAL, **77**, 1273 (1955).
(26) For a quantitative study of antibody heterogeneity, see also L. Pauling, D. Pressman and A. L. Grossberg, *ibid.*, **66**, 784 (1944).

The light scattering method of studying hapten–antibody equilibria described here stands midway between the dialysis equilibrium method, which permits a study of only monovalent-hapten systems, and the Singer–Campbell method which provides information on the equilibrium involved in the first and second antigen–antibody complex (see equation 10). This method has some advantage over the equilibrium dialysis method in that it permits investigation of the effect of the spatial arrangement of two haptenic groups on the bond formation and provides for the possibility of a study of reaction rates in which the initial and final states are clearly defined. The Singer–Campbell method has the distinct advantage of being applicable to protein antigens, but in that case an estimate of the valency of the antigen is required. In their method the equilibrium concentration of free antigen is determined by electrophoretic or ultracentrifugal resolution, and systems may be encountered in which this is made difficult by especially rapid re-equilibration among the species present in an antigen–antibody solution. The light scattering method, involving no disturbance of the equilibrium state of the solution, does not suffer from this possible limitation. While it is likely that other complications would arise in applying the light scattering method to protein antigen–antibody systems in a quantitative manner, such an effort appears nevertheless worthwhile.

Acknowledgments.—We are glad to acknowledge the gift of the haptens used in this work from Drs. D. H. Campbell and D. Pressman. We are indebted to Dr. B. M. Pitt for help in the antibody purifications, particularly for the suggestion leading to the development of Procedure B. In addition we wish to thank Dr. S. J. Singer for permission to quote the results of some of his measurements made at Yale University.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, STATE UNIVERSITY OF NEW YORK, COLLEGE OF FORESTRY]

The Role of Solvents in the Decomposition of Propionyl and Butyryl Peroxides

BY J. SMID, A. REMBAUM AND M. SZWARC

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The decomposition of propionyl and butyryl peroxides in a variety of solvents was investigated. It was shown that the primary reaction involves the rupture of the O–O bond and formation of $R\cdot CO_2$ radicals which decarboxylate rapidly, yielding the corresponding hydrocarbon radicals. It was demonstrated that butane formed in the decomposition of propionyl peroxide results from a cage recombination of ethyl radicals and that disproportionation as well as recombination takes place in the "cage." The decomposition in hydrocarbon solvents was contrasted with the decomposition proceeding in polar solvents. The role of solvents in the decomposition was discussed and the ionic mode of decomposition was considered. The radical induced decomposition was investigated, and it was demonstrated that this reaction is initiated mainly by solvent radicals.

Investigation of the pyrolysis of gaseous propionyl and butyryl peroxides[1] demonstrated that these compounds decompose in a unimolecular fashion, the rates being determined by the ease with which the respective O–O bonds are ruptured

$$R\cdot CO\cdot O\cdot O\cdot CO\cdot R \longrightarrow 2R\cdot CO_2 \qquad (1)$$

It was shown that for R = CH_3, C_2H_5 or n-C_3H_7, the activation energies and the frequency factors of reactions described by equation 1 are nearly constant, namely

	E, kcal./mole	$A \times 10^{14}$, sec.$^{-1}$
CH_3	29.5	1.8
C_2H_5	30.0	2.5
n-C_3H_7	29.6	1.9

In view of the similarity in behavior of these compounds in gaseous decomposition, the kinetics of their decomposition in a variety of solvents has been studied in an attempt to shed more light on the nature of factors which govern the rates of chemical processes taking place in solution.

(1) A. Rembaum and M. Szwarc, *J. Chem. Phys.*, **23**, 909 (1955).

Experimental and Results

Propionyl and butyryl peroxides were prepared and purified by the method described previously.[1] The following solvents were used: spectroscopically pure isoöctane, analytically pure benzene, toluene, acetic acid and acetic anhydride and chemically pure n-hexane, 1,4-dioxane, nitrobenzene and benzonitrile. All these solvents were distilled prior to use through an efficient Todd column, the middle fraction being collected. 1,4-Dioxane was distilled over calcium hydride in order to destroy the peroxides originally present in this solvent. The absence of peroxides in all the other solvents was demonstrated.

Two techniques were used in investigating the kinetics of the decomposition. One series of experiments was conducted with deaerated solutions of peroxides, sealed in glass ampoules and heated for a predetermined period of time at a desirable temperature. The temperature bath was controlled within $\pm 0.1°$, the thermometers used were calibrated by means of a standard thermometer calibrated by The National Bureau of Standards. At the end of the required period of time, the ampoules were withdrawn from thermostatic bath, cooled rapidly by immersion in the solid carbon dioxide–methanol bath, and then their contents were analyzed for undecomposed peroxide. The iodometric technique described by Wagner, Smith and Peters[2] was used in analysis of all solutions with the exception of those con-

(2) C. D. Wagner, R. H. Smith and E. D. Peters, *Ind. Eng. Chem., Anal. Ed.*, **19**, 976 (1947).