Thermodynamics of the Binding of Haptens to Rabbit Anti-2,4-dinitrophenyl Antibodies*

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ABSTRACT: The enthalpy changes in the combination of dinitrophenyl (DNP) haptens with rabbit anti-DNP antibodies and the F_{ab} fragments derived therefrom have been determined by flow calorimetry. Thermal titration of the antibodies with ϵ-*N*-DNP-L-lysine shows that about 30% of the antibody sites present bind hapten with a very large decrease in enthalpy of -22.8 ± 0.4 kcal/mole of antibody sites. This figure is independent of the site purity of the antibodies. At saturation of the antibody sites by hapten, the enthalpy decrease averaged over all sites is -15.2 ± 0.3 kcal/mole of antibody sites, if the site purity of the antibodies is assumed to be 100%, and it is more negative if the purity is less than 100%. Fluorometric titration of the antibodies leads to an enthalpy value based on the van't Hoff equation of -11 kcal/mole. If we assume this figure applies to a mole of antibody sites, we find a spread of 12 kcal/mole of antibody sites within the central 80% of the heterogeneous antibody population. This is much larger than the spread in standard free-energy changes indicated by the Sips heterogeneity index found for the antibodies by fluorometric titration.

The shape of the thermal titration curve observed with univalent F_{ab} fragments is the same as that with intact antibodies. This result indicates that the enthalpy variation exhibited by the thermal titration curves is due to site heterogeneity rather than to site interaction in the bivalent antibody molecule.

The nature of the binding of an antigen to its homologous antibody has been the subject of much study. Thermodynamic quantities characterizing several hapten–antibody and protein antigen–antibody reactions have been measured (*cf.* Singer, 1965). One important quantity, the enthalpy change of the reaction, has usually been estimated from the temperature dependence of the equilibrium constants for the reactions by use of the wan't Hoff relation. With the usual grossly heterogeneous antibodies, however, as well as for other reasons considered in the Discussion, this procedure is of only limited quantitative significance. In only one recent instance, involving the interaction of bovine serum albumin and its rabbit antibodies, has the average enthalpy change been measured directly by calorimetry (Steiner and Kitzinger, 1956). In this paper we report measurements with a sensitive flow calorimeter of the enthalpy changes accompanying the reaction of the hapten ϵ-*N*-2,4-dinitrophenyl-L-lysine (DNP-lysine)[1] and specific rabbit antibodies directed to the DNP group. The large exothermic enthalpy change of close to -23 kcal/mole of sites characterized the binding reaction of the most strongly binding 30% of the sites, indicating that a large *decrease* in entropy accompanied the binding at these sites. A broad range of ΔH values was found for the different DNP binding sites in the antibody population; this heterogeneity of values could not be detected by van't Hoff estimates of the enthalpy change in this reaction (Velick *et al.*, 1960). Significant enthalpy–entropy compensation appears to characterize the different kinds of antibody sites elicited to this hapten. In addition, a large decrease in heat capacity accompanies the binding reaction. When homogeneous antibody preparations become available in quantities sufficient for calorimetric work, further thermal titrations with haptens will be of great interest.

Materials and Methods

Antibodies. Rabbit anti-DNP antibodies were prepared by immunization with the multivalent conjugate DNP-bovine γ-gobulin, and were isolated from the pooled antisera, by previously published procedures (Farah *et al.*, 1960; Eisen, 1964). F_{ab} fragments of the purified antibodies were prepared by papain digestion following the usual procedure (*cf.* Nisonoff, 1964).

Haptens. DNP-lysine was obtained from Sigma Chemical Co., and was used without further purification. ϵ-*N*-DNP-aminocaproic acid was prepared as described by Carsten and Eisen (1953). 1-Hydroxy-2-DNP-azonaphthalene-3,6-disulfonic acid (DNPNS), purchased from Eastman Organic Chemicals Co., was recrystallized as the sodium salt. All other chemicals were of analytical grade.

Thermal titrations were performed with a flow calorimeter (Sturtevant and Lyons, 1969; Velick *et al.*, 1971) based on the Beckman Model 190B microcalorimeter. In most of the experiments, antibody solution and hapten solution were flowed into the calorimeter at equal flow rates of 0.044 ml min^{-1}, with the instrument being used at its maximum sensitivity. All appropriate corrections for viscous heating and heats of dilution were applied. With the usually employed antibody concentration (after mixing) of 25–33 μM, the heat effect observed on addition of excess hapten was an evolution of 1.4 μcal sec^{-1} or less. The temperature of the calorimeter was known to $\pm 0.1°$. All calorimetric results are expressed in kcal per *mole of binding sites*, on the *assumption of 100% purity* of the antibodies. In other words, the antibody concentration was deduced from the absorbance at 278 nm, and the site concentration was taken as twice the antibody concentration. Although as will be discussed in detail later, this procedure without doubt overestimates the site concentration, we have chosen this form for reporting our results since estimates of actual site concentrations are themselves subject to serious uncertainties. The uncertainties given for the calorimetric data are standard errors based solely on the calorimetric experiments.

In all experiments at pH 7.4 the solvent was 0.01 M phosphate containing 0.15 M NaCl, while those at pH 5.0 were conducted in 0.2 M sodium acetate buffer. Antibody concentra-

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[1] Abbreviations used are: DNP, 2,4-dinitrophenyl; DNP-lysine, ϵ-*N*-2,4-dinitrophenyl-L-lysine; DNPNS, 1-hydroxy-2-DNP-azonaphthalene-3,6-disulfonic acid.

TABLE I: Fluorescence Quenching Titration of F_{ab} Fragment from Rabbit Anti-DNP Antibody Lot 68 with DNP-lysine.

Temp (°C)	K_d^0 [a] × 10^8 (M)	a [b]	C_e [c] (μM)	Max. Quench (%)	App Site Purity (%)
4.90	1.64	0.52	0.532	79.3	53
	1.09	0.50	0.552	77.5	55
	1.15	0.54	0.545	77.3	55
25.10	4.71	0.82	0.576	74.2	58
	1.41	0.59	0.686	73.1	69
45.45	5.61	0.66	0.697	70.9	70
	7.91	0.71	0.619	69.8	62

[a] Most probable value for the binding constant. [b] Heterogeneity index, Sips distribution function. [c] Concentration of binding sites.

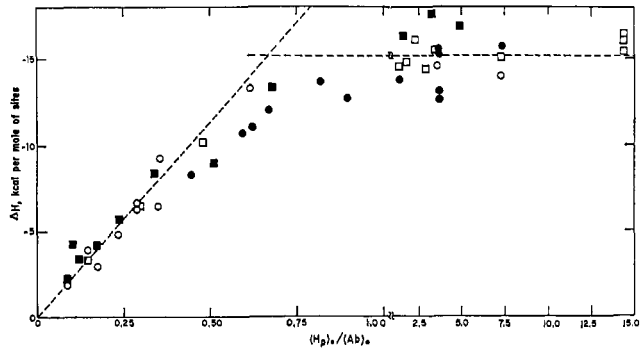

FIGURE 1: Thermal titrations of anti-DNP rabbit antibodies with ε-DNP-lysine at pH 7.4, 25°, in 0.01 M phosphate containing 0.15 M NaCl. Antibody concentrations were approximately 30 μM, and it was assumed that the antibodies were divalent. Antibody and hapten solutions were flowed at 0.044 ml/min, and the heat effect observed at hapten saturation, after correction for viscous heating and dilution heats, was approximately 90 μcal/min. (□) Antibodies from serum pool 52; (■) pool 59; (○) pool 66, first series; (●) pool 66, second series.

tions were based on an extinction coefficient of $\epsilon_{1\,cm}^{1\%} = 14.7$ at 278 nm, and concentrations of DNP-lysine on an absorptivity of 17,400 M^{-1} cm^{-1} at 360 nm.

Fluorometric titrations were carried out in cuvets with 1 × 1 cm cross-section, thermostated to ±0.1°. Protein concentrations were generally in the range 0.05–0.08 mg ml^{-1}. The fluorometer was essentially as described by Day (1963). Fluorescence was excited at 280 nm and observed at 340 nm, with spectral bandwidths of approximately 8 nm. The titration data were corrected for dilution by the added titrant, and for absorption of excitation and fluorescence radiation by free titrant. The latter corrections were based on observations of the attenuation of the fluorescence of tryptophan by the haptenic compound used in the titration, the tryptophan being used at a concentration selected to give the same absorbance at 280 nm as the antibody.

An iterative method for numerical analysis of the corrected fluorescence titration curves was developed (Barisas, 1971) which was based on the Sips isotherm (Sips, 1948; Klotz, 1953; Nisonoff and Pressman, 1958). In this expression θ is

$$\frac{\theta}{1-\theta} = \left(\frac{C}{K_d^0}\right)^a \qquad (1)$$

the fraction of antibody sites at which hapten is bound, C is the molar concentration of unbound hapten, K_d^0 is the most probable dissociation constant for an individual antibody–hapten bond, and $a \leq 1$ is the Sips index, or heterogeneity index, which describes the breadth of the distribution of values of K_d. It is assumed that fluorescence quenching is proportional to hapten binding, so that $\theta = (F_0 - F)/(F_0 - F_\infty)$, where F_0 is the initial fluorescence intensity, F is the observed intensity, and F_∞ is the intensity at maximum quenching. In application of the Sips isotherm, a third unknown parameter, in addition to K_d^0 and a, is involved, namely the concentration of antibody binding sites, $C_e = (C_t - C)/\theta$, where C_t is the total concentration of hapten. As might be expected with three independently adjustable parameters, it is not possible to obtain very reliable values for any of the parameters. This is illustrated by the duplicate titrations at 25° of a sample of F_{ab}, the derived parameters for which are given in Table I. The two experimental curves agreed within a standard deviation of 0.96%, and the individual sets of experimental data were fitted with standard deviations of 0.33 and 0.62%, respectively, but as can be seen the derived parameters are not in good agreement. This situation is unfortunately characteristic of all antibody fluorescence titration curves.

Results

Thermal Titrations. The results of thermal titrations with DNP-lysine at 25° are given in Figure 1. Four series of experiments were performed on samples of anti-DNP antibodies obtained from three completely independent pools of rabbit antisera (lots 52, 59, and 66) the last two series using independent preparations from serum 66. In computing the data, it was assumed that the concentration of binding sites is twice the apparent antibody concentration. Although this assumption is incorrect, as discussed below, it is very important to note that the enthalpy decrease per mole of hapten bound deduced from the initial slope of the plot in Figure 1 is *completely independent* of uncertainties in the site concentration, since it is actually based on the accurately known concentration of added hapten, essentially all of which is bound. The mean value for ΔH at excess hapten is, on the other hand, inversely proportional to the assumed fractional purity of the antibody. The line representing the initial slope of the titration curve was obtained by least squaring all values of ΔH for hapten-site concentration ratios ≤ 0.515 to a line passing through the origin. The line representing the level of ΔH at saturation of antibody by hapten was obtained by averaging all data for hapten-site concentration ratio ≥ 1.5. Table II gives a summary of the results of these experiments.

Additional Calorimetric Experiments. Calorimetric determinations of the heats of antibody–hapten reactions with various DNP-haptens and under altered experimental conditions were carried out with substantial excesses of the haptens. All these experiments were performed with antibody preparations derived from serum pool 66. The results are summarized in Table II.

Thermal titrations were performed on two preparations of

TABLE II: Summary of Results of Thermal Titrations of Rabbit Anti-DNP Antibody with Various DNP-haptens.

			$(Hp)_0/(Ab)_0$[a] ≤ 0.515		$(Hp)_0/(Ab)_0$[a] ≥ 1.5	
Hepten	Temp (°C)	pH	$-\Delta H$[b] ± Std Error, kcal mole^{-1}	No. of Expt	$-\Delta H$[b] ± Std Error, kcal mole^{-1}	No. of Expt
DNP-Lys[c]	25	7.4	22.8 ± 0.4	16	15.2 ± 0.3	20
DNP-Lys	5	7.4	(16.9)[f]		11.3 ± 0.1	4
DNP-Cap[d]	25	7.4			13.9 ± 0.2	4
DNP-Lys	25	5.0			12.5 ± 0.3	3
DNPNS[e]	25	5.0			11.6 ± 0.3	4

[a] $(Hp)_0$ = total hapten concentration; $(Ab)_0$ = total concentration of antibody binding sites *assuming 100% purity*. [b] kcal per mole of binding sites. [c] ε-*N*-DNP-lysine. [d] ε-*N*-DNP-aminocaproate. [e] 1-Hydroxy-2-dinitrophenylazonaphthalene-3,6-disulfonate. [f] Calculated from the mean ΔH at saturation assuming the same ratio of ΔH's as observed with DNP-Lys at 25°, pH 7.4. This value gives $\Delta C_p = -300 \pm 30$ cal deg^{-1} mole^{-1}.

TABLE III: Summary of Results from Fluorescence Titration of Rabbit Anti-DNP Antibody and Corresponding F_{ab} Fragments with DNP-lysine.

Material Titrated	Buffer	pH	Temp (°C)	Log K_d^0[a] (M)	a[b]	Mean Site Purity (%)	ΔH^0[c] (kcal mole^{-1})
Lot 66 Ab	Phosphate	7.4	6.2	−8.25	0.33	60	
			25.0	−7.77	0.54		11
			46.8	−7.13	0.59		
Lot 66 Ab	Acetate	5.0	5.3	−8.25	0.42	60	
			25.4	−7.74	0.59		11
			46.7	−7.13	0.76		
Lot 68 Ab	Phosphate	7.4	5.3	−8.00	0.73	66	
			25.0	−7.78	0.73		8
			45.1	−7.19	0.87		
Lot 68 F_{ab}	Phosphate	7.4	4.9	−7.89	0.52	60	
			25.1	−7.59	0.70		7
			45.5	−7.18	0.68		

[a] Most probable value for the binding constant. [b] Heterogeneity index, Sips distribution function. [c] Calculated from the temperature variation of log K_d^0 according to the van't Hoff equation.

univalent F_{ab} fragments (Porter, 1959), one derived from antibody lot number 64 and the other from lot 68. The titrations gave, on the basis of a molecular weight of 50,000, initial slopes of −19 and −16 kcal per mole, respectively, and enthalpy values at saturation by hapten of −12 and −8 kcal per mole, respectively. These values are considerably less reliable than those obtained by titration of intact antibody. Figure 2 gives a normalized plot of the F_{ab} titration data.

Fluorescence Quenching Titrations. A typical fluorescence titration curve is given in Figure 3. A solution (3 ml) containing 0.0501 mg ml^{-1} of F_{ab} fragments obtained from lot 68 anti-DNP antibodies was titrated at 25° with 10^{-4} M DNP-lysine (first 0.05 ml) and then with 2×10^{-3} M DNP-lysine (succeeding 0.08 ml). The fluorescence intensity in arbitrary units is plotted against volume of titrant added. The experimental points are represented by filled circles, and the solid curve is calculated using the final values of C_e, K_d^0, and a, arrived at as described above, together with the nonspecific attenuation due to the added hapten as evaluated with tryptophan. The open circles are corrected for dilution and for this nonspecific attenuation.

The results obtained for all the titrations performed on the F_{ab} preparation are summarized in Table I. Table III gives the mean values for the parameters obtained from duplicate or triplicate titrations of intact antibody preparations, as well as the mean values for the parameters listed in Table I for the F_{ab} preparation.

Discussion

Thermal Titrations. The calorimetric data appear to be quite reliable as judged by the reproducibility of the results obtained with purified antibodies prepared from independent pools of antisera. Thus 8 experiments with antisera pools 52 and 59 gave an initial slope for the titration curve only 8% larger than that found for 8 experiments with antiserum pool 66.

Enthalpy of Binding of DNP-lysine at 25°. The initial slope of the calorimetric titration curve in Figure 2 gives a value for the enthalpy of binding of DNP-lysine to the antibodies which is completely independent of the concentration of antibody sites. This value, −22.8 ± 0.4 kcal per mole of hapten bound, can be considered as a firmly established quantity representative of high-affinity anti-DNP rabbit antibodies. Eisen and Siskind (1964) and McGuigan and Eisen (1968), as well as

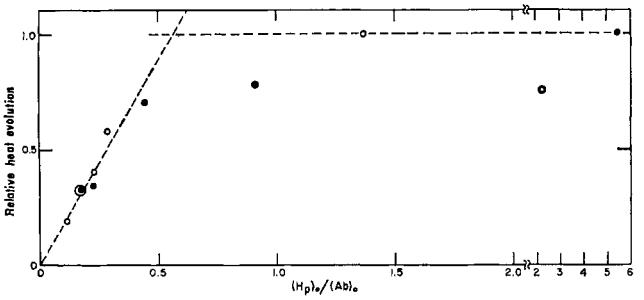

FIGURE 2: Normalized plot of the thermal titrations of two preparations of F_{ab} fragments with ϵ-DNP-lysine at pH 7.4, 25°, in 0.01 M phosphate containing 0.15 M NaCl. (●) F_{ab} prepared from serum pool 64; 45 μM; (○) F_{ab} prepared from serum pool 68; 53 μM.

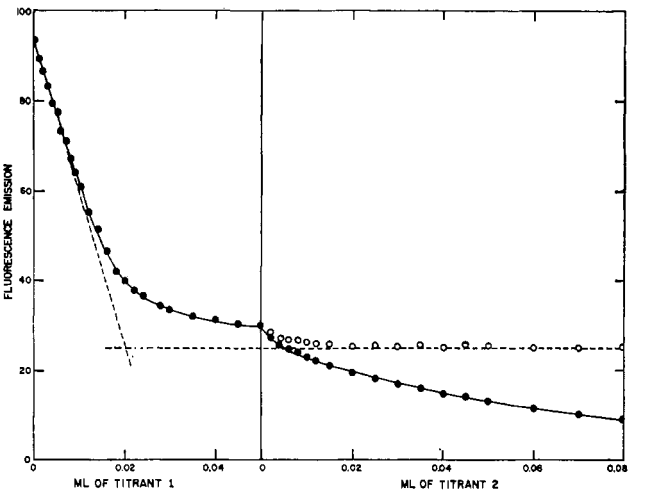

FIGURE 3: Fluorescence titration of 3.0 ml of a 1.0 μM solution of F_{ab} fragment (serum pool 68) at pH 7.4, 25° in 0.01 M phosphate buffer containing 0.15 M NaCl. Titrant 1 was 10^{-4} M ϵ-DNP-lysine, and titrant 2 was 2×10^{-3} M hapten. Filled circles are the uncorrected observed values; open circles are values corrected for dilution and nonspecific attenuation (see text). The solid curve is calculated using the parameters deduced from analysis of the titration data in terms of the Sips distribution function. Fluorescence intensity is given in arbitrary units.

other authors, have reported large differences between the thermodynamic properties of the low-affinity antibodies obtained after brief periods of immunization and those of the high-affinity antibodies resulting from longer periods of immunization, and it is quite possible that similar differences in calorimetrically determined binding enthalpies would be observed.

The magnitude of the enthalpy decrease accompanying the binding of DNP-lysine to anti-DNP antibodies is surprising, in that none of the currently postulated sources of binding free energy would be expected to lead to such a large change in enthalpy.

The standard free-energy change associated with hapten binding at the 30% of sites which exhibit the tightest binding is probably no more negative than −12 kcal/mole. It thus appears that binding at these sites is accompanied by an entropy *decrease* of at least 35 cal deg^{-1} $mole^{-1}$.

Since the enthalpy observed at saturation of the antibody sites with hapten is considerably smaller than that for the first additions of hapten, it would appear that there is a large heterogeneity with respect to binding enthalpy. Quantitative interpretation of the data in terms of heterogeneity is, however, difficult because of uncertainty in the antibody site concentration. For example, the antibody from serum pool 66 after 2-weeks dialysis against buffer had an absorption at 358 nm equal to 0.017 times that at 278 nm; interpreted in terms of irreversibly bound 2,4-dinitrophenol (ϵ_{358} 1.49×10^4 M^{-1} cm^{-1} at pH 7.4), resulting from its use in the preparation of the antibodies, this corresponds to blocking of 13% of the antibody sites. A similar residue of dinitrophenol was reported by Farah *et al.* (1960) after utilization of [^{14}C]dinitrophenol in the preparative procedure. Furthermore, the fluorescence titrations indicate a site concentration only about 1.4 times the molar antibody concentration. If the saturation value for the binding enthalpy, −15.2 ± 0.3 kcal/mole of sites, which is based on the assumption of 100% purity of the antibodies and 2 sites/molecule, is increased by the factor 2/1.4, it becomes −21.7 kcal/mole of sites. This value is so close to the initial value that it leaves no room for the heterogeneity in binding enthalpies which seems to be clearly indicated by the van't Hoff enthalpies derived from the fluorescence titrations. It therefore seems most reasonable to conclude that the fluorescence titrations underestimate the site concentration, and that the antibody purity is approximately 87%, as indicated by the spectral data for the preparation, and as reported for other preparations by Farah *et al.* (1960) and others. A minor difficulty with this conclusion is that application of the factor 1/0.87 to the titration curve in Figure 1 raises the hapten–antibody site ratio at which the observed enthalpy becomes equal to the saturation value to 1.15. The fluorometrically estimated value for K_d^0 is so small that, even allowing for the possibility that the binding sites with low binding enthalpies may also have low binding constants, the reaction at the high concentrations used in the calorimetric work should be at least 95% complete at all hapten–antibody ratios. It would thus be expected that the titration curve would be horizontal at the apparent equivalence point. However, the precision of the data is not such as to make this a very serious difficulty.

The possibility of nonspecific binding of hapten, which equilibrium dialysis experiments at lower concentrations (Eisen, 1964) failed to detect, was investigated calorimetrically. It was found that the enthalpy change on adding a large excess of DNP-lysine to bovine γ-globulin at a concentration of 5 mg ml^{-1} was 0 ± 1 kcal/mole of protein.

The mean value for the enthalpy of binding, $\Delta H = -(15.2/0.87) = -17.5$ kcal/mole of sites, is not necessarily inconsistent with the small values reported here and elsewhere for the van't Hoff enthalpy (*cf.* Table III). As is always the case with enthalpy changes obtained by the van't Hoff method, there is nothing in the equilibrium measurements which gives any information as to the size of the mole to which the enthalpy change refers. This is because $d \ln K/dT$ is obviously independent of the units of K. However, since the smallest unit of reaction in the present case is one binding site plus one ligand molecule, we may conclude that the *maximum* value for the enthalpy change per mole of sites derivable from the equilibrium measurements is about −11 kcal. If this value is ascribed to the last 0.3 of the binding sites occupied in the titration,[2] and −22.8 kcal/mole of sites to the first 0.4 of

[2] Enthalpy changes obtained by the van't Hoff method are standard state quantities. Within the usual (necessary) approximation of assuming all activity coefficients equal to unity, ΔH° is in general independent of the choice of standard states, and can be directly compared with calorimetric ΔH's determined at low concentrations.

the sites occupied, then a value of −17 kcal/mole of sites for the remaining sites leads to the observed average value.

It should be noted that an enthalpy spread from −11 to −23 kcal per mole of sites in the absence of any entropy compensation would correspond to a range of values of K_d for as small a portion of the distribution as the central 70% of approximately 9 powers of 10. Since this represents a far broader distribution of values of K_d than indicated by the fluorescence experiments, we must conclude that there is much enthalpy–entropy compensation, that is, that sites having more negative binding enthalpies tend to have more negative binding entropies (Lumry and Rajender, 1970).

Measurements of the binding of DNP-lysine at pH 5.0 were made at saturating concentrations of the hapten (Table II). The enthalpy change at this pH is 75% of that at pH 7.4. Since the hapten is zwitterionic at both pH levels, the marked decrease in binding enthalpy is to be attributed to some change in the antibody protein. It is interesting that ϵ-DNP-aminocaproate, which carries a full negative charge, shows a binding enthalpy at pH 7.4 only 9% less than that observed with DNP-lysine at the same pH, which suggests that electrostatic interactions have but little effect on the binding enthalpy.

Heat Capacity Change Due to Hapten Binding. DNP-lysine, at saturating concentrations, binds to the antibodies at pH 7.4 and 5° with a decrease in enthalpy of 11.3 kcal/mole of sites (Table II). If we assume that the ratio of this figure to the value which would have been observed for the first 0.3 equiv of hapten bound is the same as at 25°, we obtain $\Delta H = -16.9$ kcal/mole of sites for the initial enthalpy change. This leads to a value of $\Delta C_p = -300 \pm 30$ cal deg^{-1} per mole of sites for the apparent change in heat capacity of the solutes resulting from binding at the strongest binding group of sites. The average value of ΔC_p, assuming as before 87% purity for the antibody, is -220 ± 20 cal deg^{-1} per mole of sites.

The large magnitude of ΔC_p is a recurring feature of the binding of small ligands to proteins. For example, Hinz *et al.* (1971) found $\Delta C_p = -410$ cal deg^{-1} per mole of ligand bound for the binding of the inhibitor hexitol 1,6-diphosphate to muscle aldolase, and Velick *et al.* (1971) reported $\Delta C_p = -520$ cal deg^{-1} per mole of ligand bound for the binding of NAD to yeast glyceraldehyde 3-phosphate dehydrogenase. Although it is not possible at present to discuss in quantitative manner the origin of such large decreases in apparent heat capacity, two factors may be mentioned as probably of leading importance. (1) If the exposure of nonpolar groups to the solvent is decreased as a result of the binding, the heat capacity would be expected to decrease since bulk water has a lower heat capacity than the structured water surrounding exposed nonpolar groups. (2) It is not unreasonable to expect that both the ligand and the protein lose internal easily excited degrees of freedom, such as rotations or torsional vibrations about single bonds, as a result of ligand binding. The importance of this latter factor is perhaps further indicated by the entropy decrease accompanying hapten binding which was noted above.

It is well to point out that, whatever the origin of these large heat capacity changes, they have an importance resulting simply from the fact that they entail large temperature dependences for ΔH and ΔS, by virtue of the fundamental relations

$$\left(\frac{\partial \Delta H}{\partial T}\right)_P = \Delta C_p$$

$$\left(\frac{\partial \Delta S}{\partial T}\right)_P = \frac{\Delta C_p}{T}$$

Binding of DNPNS. As listed in Table II, the enthalpy of binding of DNPNS at 25° and pH 5.0 is only 7% less than that of DNP-lysine under the same conditions. This result, in view of the difference in structure between this hapten and DNP-lysine, suggests that the enthalpy of binding may be largely determined by the dinitrophenyl group itself.

Binding of DNP-lysine to F_{ab} Fragments. Two independently prepared samples of F_{ab} fragments, derived from antiserum pools 64 and 68, were titrated with DNP-lysine. The first series of experiments (filled circles in Figure 3) gave an initial $\Delta H = -18.9$ kcal/mole of sites and at saturation an average $\Delta H = -12$ kcal/mole of sites, while the second series (open circles in the figure) gave, respectively, −15.9 and −8.3 kcal per mole of sites. The reason for the poor agreement between these series is not known. The shape of the titration curves is shown in Figure 3, in which the thermal data have been normalized; it is seen to be generally similar to that observed with intact antibodies. We may thus conclude that the univalent fragments are about as heterogeneous with respect to binding enthalpies as are the intact antibodies. The fluorescence titrations show that this is also the case with respect to binding constants, as has been previously demonstrated (Nisonoff *et al.*, 1960).

Fluorescence Titrations. The experimental points for the second titration of F_{ab} at 25° (Table I) are given in Figure 3, together with the curve calculated by the Sips eq 1 using the parameters given in Table I. The standard deviation of the points, 0.33% of F_0, is well within experimental uncertainty. This good fit indicates that no better set of values for C_e, K_d^0, and a could be obtained by any other method of treating the titration data. At the same time, it cannot be concluded that the closeness of fit is a proof that the Sips distribution is uniquely appropriate for antibody systems.

The open circles in Figure 3 are corrected for the nonspecific attenuation due to the large amounts of hapten added. These corrected values reach a maximum quenching level of 73.1%. The intersection of the horizontal line corresponding to this quenching with a line having the initial slope of the titration curve gives a graphical measure of $C_e = 0.672$ μM which agrees well with the value obtained by the calculational procedure outlined above.

The concentration of protein in the titration was 0.0497 mg ml^{-1} at the equivalence point. If we take a molecular weight of 50,000 (Porter, 1959) for the F_{ab} fragment, and assume no other protein present, we conclude that only 69% of the F_{ab} molecules have sites which bind DNP-lysine. The maximum quenching obtained with F_{ab} fragments has been reported by others (Little and Eisen, 1968) to be higher than listed in Table I. Although the reason for this discrepancy is unknown, it seems unlikely that the shape of the F_{ab} thermal titration curve (Figure 2) is seriously in error.

The reproducibility of the fluorometric titrations is indicated by the variations between replicate titrations shown in Table I. We estimate overall uncertainties in the titration results listed in Table II as follows: log K_d^0, ±0.15; a, ±0.1.

It is evident in Table III that changing the pH from 7.4 to 5.0, with a change in buffer from phosphate to acetate, had no effect on K_d^0, and very little if any on the heterogeneity index (*cf.* Velick *et al.*, 1960).

Estimates of the enthalpies of binding were made using the van't Hoff expression, assuming no variation of ΔH° with temperature. The values in the last column of Table III were obtained in this way.

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Structural Units of Canine Serum and Secretory Immunoglobulin A*

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ABSTRACT: Immunoglobulin A was isolated from canine serum and compared to canine secretory IgA (colostral) to clarify molecular weight and antigenic differences. Serum and secretory IgA differ in molecular weight by approximately 75,000 and have sedimentation coefficients of 10 and 11.7 S, respectively; yet disulfide reduction decreased the sedimentation coefficient of both molecules to approximately 7 S. Gel filtration of unreduced secretory IgA in 6 M guanidine-HCl dissociated 13% of the molecule in a manner analogous to the dissociation of rabbit colostral IgA. A portion of this dissociated protein, which was approximately the size of γ chains, possessed antigenic determinants specific for secretory IgA which were localized to the Fc_α fragment produced by tryptic digestion. The presence of a secretory piece-like protein on canine secretory IgA seems certain, although it was not identified specifically in polyacrylamide gels. It is proposed that both serum and secretory IgA molecules consist of two covalently linked subunits, each composed of two α and two light chains. While the greater mass of canine secretory IgA may be due in small part to a slightly larger α chain, most of the extra mass can be accounted for by the attachment of an additional but as yet incompletely characterized component which is similar to the secretory piece found in humans and rabbits.

Johnson and Vaughan (1967) described an immunoglobulin in canine serum, salivary, and bronchial secretions, called intermediate Sγ1, which they thought was analogous to IgA. Immunoprecipitation reactions discussed in that study suggested that antigenic differences existed between the serum and secretory IgA molecules and that these differences were lost after reduction and alkylation of the salivary protein. Subsequently, Vaerman and Heremans (1968) demonstrated immunologic homology between canine intermediate Sγ1 present in serum and external fluids and IgA from similar human fluids through shared antigenic determinants which were recognized by a group of rabbit anti-human IgA antisera. Additional work by Vaerman and Heremans (1969, 1970) revealed that disulfide reduction and alkylation of canine serum and milk whey immunoglobulins changed the sedimentation coefficient of the IgA proteins from 11 to 7 S, suggesting that the parent molecules consisted of two covalently linked subunits.

Canine serum IgA exists principally as a large molecule which is distinctly different in its sedimentation behavior from the 7S serum IgA of humans and rabbits, although polymeric forms of serum IgA have been found in small amounts in these species (Tomasi and Bienenstock, 1968). Because antigenic differences have been difficult to detect consistently between canine serum and secretory IgA, the possibility existed that secretory IgA might not have the extra protein component, designated secretory or transport piece, which is attached to secretory IgA in humans and rabbits.

In this report an isolation method for canine serum IgA is

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