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Thermodynamics of the Binding of 2,4-Dinitrophenyl and 2,4,6-Trinitrophenyl Haptens to the Homologous and Heterologous Rabbit Antibodies†

B. G. Barisas, S. J. Singer, and J. M. Sturtevant*

ABSTRACT: The binding of ε-*N*-2,4,6-trinitrophenyl(TNP)-L-lysine to rabbit anti-TNP antibody at 25° is accompanied by an enthalpy decrease of 21.7 ± 0.4 kcal per mole of ligand bound and a decrease in apparent heat capacity of −155 cal deg^{-1} mole^{-1}. Thermal titration curves, both for this system and for the concurrently examined binding of ε-*N*-2,4-dinitrophenyl(DNP)-L-lysine to rabbit anti-DNP antibody, indicate no heterogeneity in the binding enthalpies. The fact that the standard van't Hoff enthalpy deduced from fluorescence titrations is only half the above magnitude is surprising in view of the lack of any calorimetric indication of heterogeneity. The conclusion in a previous paper that an anti-2,4-dinitrophenyl antibody exhibited marked heterogeneity of binding enthalpy is made doubtful by these observations. For both anti-DNP and anti-TNP antibodies the free energy of binding the heterologous hapten is 2–3 kcal mole^{-1} less favorable than that for the homologous hapten. Thermal data show that this is entirely an enthalpy effect.

In a recent paper (Barisas *et al.*, 1971) we reported calorimetric determinations of the enthalpy of binding of various dinitrophenyl (DNP)[1] haptens to anti-DNP rabbit antibodies. The results obtained with antibodies from three different pooled antisera indicated that for at least the tighter binding part of the population of antibody sites the binding enthalpy is −22.8 ± 0.4 kcal per mole of sites. In view of the unexpectedly large magnitude of this enthalpy change and the difficulty of interpreting it in terms of current views of noncovalent intermolecular interactions, it was decided to extend our calorimetric observations. We report here enthalpy data for the reactions of both ε-*N*-DNP-lysine and ε-*N*-2,4,6-trinitrophenyl-L-lysine (TNP-lysine) with anti-DNP and anti-TNP rabbit antibodies. In our previous publication it was tentatively concluded that the anti-DNP antibodies exhibited a large heterogeneity with respect to binding enthalpies, since the van't Hoff value for the enthalpy, obtained from fluorescence titrations over a range of temperature, was much smaller than the calorimetric enthalpy. It was hoped that further insight into this matter might be forthcoming from further thermal titrations.

Materials and Methods

Antibodies. Rabbit anti-DNP and anti-TNP antibodies were prepared by immunization with the multivalent conjugates DNP- and TNP-bovine γ-globulin, respectively. The antibodies were purified by precipitation by *cross-reacting* antigens, TNP-bovine serum albumin in the case of the anti-DNP antibody, and DNP-bovine serum albumin in the case of the anti-TNP antibody. The precipitates were solubilized by dinitrophenol or trinitrophenol, and the solubilizing hapten was removed by ion-exchange chromatography (Farah *et al.*, 1960; Eisen, 1964). The antibodies were shown to have a high degree of purity by fluorescence titration with the homologous haptens, the anti-DNP antibody being 96% titratable, and the anti-TNP material 92% titratable, based on antibody concentrations determined using extinction coefficients of $\epsilon_{278}^{1\%}$ 15.8 cm^{-1} for anti-TNP antibodies and $\epsilon_{278}^{1\%}$ 16.4 cm^{-1} for anti-DNP antibodies (Little and Eisen, 1968), and a molecular weight for rabbit γ-globulin of 145,000 (Marler *et al.*, 1964). After prolonged dialysis, absorption measurements indicated that in the case of the anti-DNP antibody 8%

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520, and the Department of Biology, University of California, San Diego, La Jolla, California 92037. *Received March 1, 1972.* This work was aided by Research Grants AI-06659 (S. J. S.) and GM-04725 (J. M. S.) from the National Institutes of Health of the U. S. Public Health Service, and GB-23545 (J. M. S.) from the National Science Foundation.

* Author to whom correspondence should be addressed at Yale University.

[1] Abbreviations used are: DNP, 2,4-dinitrophenyl; DNPAC, ε-*N*-2,4-dinitrophenylaminocaproic acid; DNP-lysine, ε-*N*-2,4-dinitrophenyl-L-lysine; TNP, 2,4,6-trinitrophenyl; TNPAC, ε-*N*-2,4,6-trinitrophenylaminocaproic acid; TNP-lysine, ε-*N*-2,4,6-trinitrophenyl-L-lysine.

TABLE I: Thermal Titrations of Rabbit Anti-DNP (D74P) and Anti-TNP (T1 + 2P) Antibodies with DNP-Lysine and TNP-Lysine.

Antibody Lot No.	Hapten	Temp (°C)	Method of Evaluation	ΔH_b (kcal mole^{-1})	ΔC_p (cal deg^{-1} mole^{-1})
D74P	DNP-Lys	25	Initial slope	−16.1 ± 1.2	
			Saturation	−14.1 ± 0.7	
	TNP-Lys	25	Saturation	−10.2 ± 0.6	
T1 + 2P	TNP-Lys	5	Initial slope	−18.6 ± 0.5	
					−155 ± 31
		25	Initial slope	−21.7 ± 0.4	
		5	Saturation	−16.7 ± 0.4	
					−185 ± 22
		25	Saturation	−20.4 ± 0.2	
	DNP-Lys	5	Saturation	−13.1 ± 0.6	
					−205 ± 52
		25	Saturation	−17.1 ± 0.6	

of the sites were irreversibly occupied by solubilizing hapten, and 4% in the case of the anti-TNP antibody.

Haptens. DNP-lysine was obtained from Sigma Chemical Co., and was used without further purification. TNP-lysine was prepared as described by Okuyama and Satake (1960) and had an uncorrected melting point of 196–197°. Hapten concentrations were determined using the molar extinction coefficients 17,400 cm^{-1} at 360 nm for DNP-lysine (Carsten and Eisen, 1953) and 15,400 cm^{-1} at 348 nm for TNP-lysine (Little and Eisen, 1968).

Thermal and fluorometric titrations were performed as described earlier (Barisas *et al.*, 1971). All experiments were performed in 0.01 M phosphate containing 0.15 M NaCl, pH 7.4 (measured at 25°).

Results

Thermal Titrations. The results of thermal titrations of anti-DNP antibody with DNP-lysine at 25° are shown in Figure 1, and of anti-TNP antibody with TNP-lysine at 5° and 25° in Figure 2. Antibody concentrations are site concentrations based on absorption at 278 nm, assuming two sites per molecule, uncorrected for the deviation from 100% purity mentioned above.

Two independent estimates of ΔH_b, the molar enthalpy of binding of hapten, can be obtained from each of the titration curves: the initial slope of the curve, in the tight binding systems studied here gives a value of ΔH_b which depends on the hapten purity but not at all on the antibody purity; and the limiting horizontal portion of the curve gives an estimate which depends on the antibody purity. These various estimates are summarized in Table I. The uncertainty estimates are standard errors of the mean values.

Additional Calorimetric Experiments. Insufficient amounts of antibodies were available to permit thermal titrations with the heterologous haptens, so that values of ΔH_b for TNP-lysine plus anti-DNP antibody and for DNP-lysine plus anti-TNP antibody were determined only in the presence of saturating concentrations of the haptens. The enthalpies obtained in this way, corrected for the fraction of sites irreversibly occupied by solubilizing hapten, together with those from the saturating experiments with homologous haptens, similarly corrected for the residual binding of solubilizing haptens, are listed in Table I.

Fluorescence titrations were carried out for a number of systems, as indicated in Table II. The experimental titration curves were treated mathematically according to the Sips distribution function (Sips, 1948; Klotz, 1953; Nisonoff and Pressman, 1958) as outlined earlier (Barisas *et al.*, 1971;

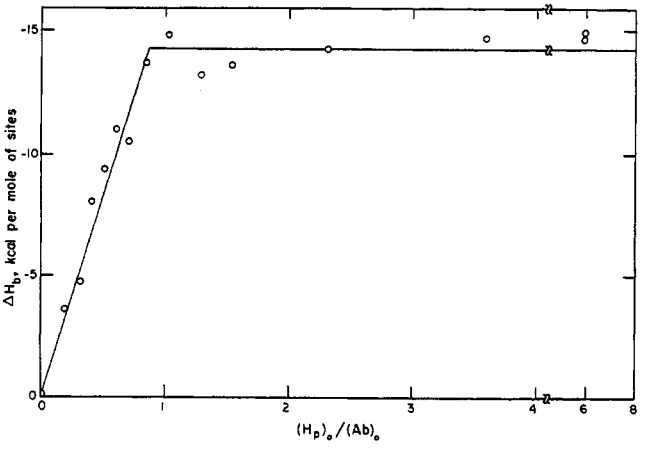

FIGURE 1: Thermal titration of D74P anti-DNP rabbit antibody with DNP-lysine at pH 7.4, 25°.

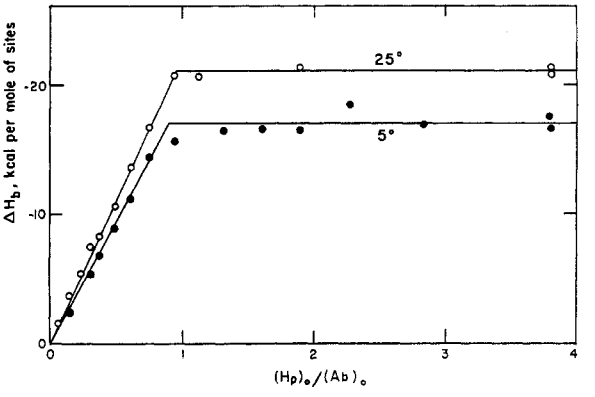

FIGURE 2: Thermal titrations of T1 + 2 anti-TNP rabbit antibody with TNP-lysine at pH 7.4, 5° and 25°.

TABLE II: Fluorescence Titration of Rabbit Anti-TNP and Anti-DNP Antibodies at Various Temperatures.

Antibody Lot No.	Hapten	Temp (°C)	Log K_d°[a] (M)	a[b]	$-\Delta H$°[c] (kcal mole^{-1})
T1 + 2P	TNP-Lys	5	−9.45	0.57	
		25	−8.98	0.57	−10
		45	−8.43	0.57	
T1 + 2P	DNP-Lys	5	−7.66	0.71	
		25	−7.27	0.71	−10
		45	−6.66	0.71	
D74P	DNP-Lys	25	−7.96	0.71	
D74P	TNP-Lys	25	−6.70	0.57	

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

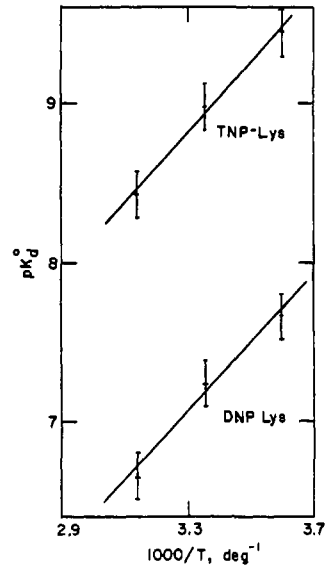

FIGURE 3: van't Hoff plots for the binding of TNP-Lys and DNP-Lys by T1 + 2P rabbit anti-TNP antibody. The antibody concentration was 0.0400 mg/ml, and the hapten solutions used as titrants were 10^{-4} M and 2×10^{-3} M, respectively.

Barisas, 1971), including evaluation of the standard enthalpy changes by means of the van't Hoff equation. van't Hoff plots for the anti-TNP antibody are given in Figure 3. Since no statistically significant curvature is visible in these plots, it was assumed that the standard enthalpy of binding is independent of temperature. The results of these experiments are included in Table II.

Discussion

Calorimetric Binding Enthalpies. The enthalpy of binding of DNP-lysine to anti-DNP antibody found in the present thermal titration is markedly lower than that observed earlier (Barisas *et al.*, 1971) with 3 different preparations of antibody (lots 52, 59, and 66). The antiserum from which antibody D74P, used in the present work, was obtained had a rather low titer of 0.84 mg/ml and gave a very low yield (20%) of purified antibody. In all other respects D74P behaved normally. Whether the significantly different enthalpy noted here is representative of the variability of antibody preparations or is due to some abnormality in D74P cannot be decided.

The enthalpy of binding observed in the TNP-lysine–anti-TNP antibody system is of nearly the same magnitude as reported earlier (Barisas *et al.*, 1971) for the DNP system, −21.7 kcal mole^{-1} as compared with −22.8 kcal mole^{-1}. The decrease in the apparent heat capacity accompanying the binding process is 155 cal deg^{-1} mole^{-1}, as compared with 300 cal deg^{-1} mole^{-1} observed for the DNP system (Barisas *et al.*, 1971).

With each antibody the enthalpy of binding the heterologous hapten is significantly less negative than that for the homologous hapten. Inspection of the data in Table III shows that the decreased tightness of binding of heterologous haptens is entirely an enthalpy effect; with both antibodies, the entropy change is actually less unfavorable with the heterologous hapten.

As pointed out in our earlier paper, it is not possible to give interpretations of the calorimetric results in terms of currently postulated sources of binding energies. The very large enthalpy decreases, which lie in the lower part of the range of covalent bond energies, are particularly difficult to understand. The small hapten molecules do not appear to provide the possibility of a cooperative summation of several noncovalent bonds.

The entropy decreases calculated for the anti-DNP antibody are roughly equal to the cratic entropy change for a reaction of the type A + B = C (−8 cal deg^{-1} mole^{-1}), but those for the anti-TNP antibody are much more negative.

Heterogeneity of Binding Enthalpies. In our earlier work (Barisas *et al.*, 1971), we concluded that the anti-DNP antibody employed was much more heterogeneous with respect to binding enthalpies than to binding free energies. This conclusion was largely based on the small value of the binding enthalpy evaluated from fluorescence titration data by the van't Hoff method, and a quite uncertain estimate of the purity of the antibody. It now appears that this conclusion may be incorrect, at least for the antibody preparations used in our present work. This is most clearly seen in the case of the binding of TNP-lysine to anti-TNP antibody. This antibody appeared to be 92% pure on the basis of fluorescence titration, and 96% pure as judged by the amount of irreversibly bound solubilizing hapten indicated by absorption at 356 nm. The titration curve with TNP-lysine at 25° (Figure 2)

TABLE III: Thermodynamic Functions for the Binding of DNP-lysine and TNP-lysine to Rabbit Anti-DNP (D74P) and Anti-TNP (T1 + 2P) Antibodies at 25°.

Antibody Lot. No.	Hapten	ΔG° (kcal mole^{-1})	ΔH (kcal mole^{-1})[a]	ΔS° (cal deg^{-1} mole^{-1})
D74P	DNP-Lys	−10.8	−15.3	−15.1
	TNP-Lys	−9.1	−11.1	−6.7
T1 + 2P	DNP-Lys	−9.9	−17.8	−26.5
	TNP-Lys	−12.2	−21.3	−30.5

[a] Corrected for antibody sites rendered inactive by the presence of irreversibly bound solubilizing antigen.

showed a very sharp break at the equivalence point, and the saturation enthalpy, after correction for impurity, agreed well with the value obtained from the initial slope of the titration curve. Thus the calorimetric data give no indication of heterogeneity with respect to binding enthalpy. It must be concluded either that this antibody preparation has a spread of enthalpy values much smaller than found for the free energy by fluorescence titration, or that there is no correlation between the values for ΔH_b and ΔG_b, with sites having any particular ΔH_b being uniformly distributed over all possible classes of ΔG_b. If the latter possibility is the correct one, we have here an extreme case of enthalpy–entropy compensation.

It is not at all obvious how the low value for the van't Hoff enthalpy of binding, less than half the calorimetric value, is to be accounted for. Both values clearly refer to the binding of 1 mole of ligand. The standard error of the least-squared slope of the van't Hoff plot corresponds to ± 2 kcal mole^{-1} in ΔH_b°. A possible source of the discrepancy is a systematic error in the dissociation constant. This could arise, for example, from a correlation such as suggested by McGuigan and Eisen (1968) of high binding affinity with high tryptophan content in antibody molecules. It is also possible that the Sips distribution function does not give a proper representation of the heterogeneity of binding free energies at high fractional extents of binding.

In view of the fact that the thermodynamic data for the systems studied in this work may well be atypical, it is important that accurate thermal data be obtained for a wide variety of antibody–hapten or antibody–antigen systems.

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Human Secretory Component. Comparison of the Form Occurring in Exocrine Immunoglobulin A to the Free Form†

Michael E. Lamm* and Jacob Greenberg

ABSTRACT: The free and bound forms of secretory component have been isolated from human colostrum and characterized. Free secretory component was isolated by conventional techniques. The finding that free secretory component lacks methionine formed the basis for a method of isolating bound secretory component, which could be purified from reduced and alkylated secretory immunoglobulin A by gel filtration in 4 M guanidine hydrochloride following reaction with cyanogen bromide. Free and bound secretory components are similar with respect to antigenic determinants, molecular weight (71,000) and composition.

In recent years there has been much interest in the immune mechanisms of mucous membrane secretions, with particular attention toward their role as a first-line defense system whose properties may under certain circumstances be independent of circulating antibody. Unlike serum, where immunoglobulin G (IgG) is the predominant immunoglobulin class, in mucous membrane secretions immunoglobulin A (IgA) predominates, and in a number of situations local immunity in secretions has been shown to be mediated by IgA (Tomasi and Bienenstock, 1968; Dayton *et al.*, 1969).

In addition to quantitative differences between the IgA content of serum *vs.* secretions, IgA in secretions has distinctive structural features. Besides the H (α) and L chains of serum-type IgA, most of which occurs in molecules with a sedimentation coefficient of 7 S, the IgA in secretions occurs predominantly as an 11S molecule and has an additional component termed the secretory component (SC),[1] whose function has not been established (Tomasi *et al.*, 1965; Tomasi and Bienenstock, 1968). Recently an additional moiety, the J chain (or F component), has been described in polymeric IgA (Halpern and Koshland, 1970; Mestecky *et al.*, 1971; O'Daly and Cebra, 1971b). The J chain is not secretion specific nor is it peculiar to IgA since it also occcurs in IgM.

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[1] Abbreviations used are: SC, secretory component; BSC, bound secretory component; FSC, free secretory component; Gdn·HCl, guanidine hydrochloride; CFA, complete Freund's adjuvant.