THERMODYNAMICS OF HAPTEN-ANTIBODY INTERACTIONS

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I. INTRODUCTION

A combination of thermodynamic and structural information is necessary for complete understanding of the mechanism(s) of antigen-antibody interaction(s) and hence, immunological specificity. In a functional sense, it would be highly desirable to unravel the mechanism(s) involved in the interaction of antibodies with complex polyvalent antigens as models for those likely to be encountered in nature such as in various disease processes of bacterial, viral, and parasitic origin. However, one of the most serious problems encountered in such studies stems from the nonspecific factors that affect the extent of the reaction of the antibody with the antigenic determinant and becomes most obvious when precipitation is involved in the reaction under investigation. In these cases, although it might be possible to obtain some thermodynamic information from the solubilities of the specific precipitates formed, the latter depending not only on the free energy for the formation of specific antigenic determinant-antibody complexes, but also on the free energy change owing to the transfer of protein molecules from solution into solid phase, the law of mass action does not apply to such complex secondary reactions. Therefore, in order to avoid difficulties in interpretation, various thermodynamic studies have been carried out employing hapten-antibody reactions as model systems. Whereas two studies reported have employed calorimetry to obtain thermodynamic information on the interaction of a polyvalent antigen, bovine serum albumin with specific antibody,[1,2] another one has recently employed precipitation in tubes at optimal ratios.[75] Although the kinetics of hapten-antibody interaction(s) have been well reviewed,[3] the thermodynamics of hapten- and antigen-antibody interaction(s) had escaped scrutiny for nearly two decades until recently[4] when the current status of the thermodynamic mechanism of hapten-antibody interactions was briefly summarized. In this review, I have attempted to present in some detail the current concepts that have emerged within the past few years on the thermodynamic mechanism(s) of hapten-antibody interaction(s) and the methods employed to procure such information.

II. POSSIBLE THERMODYNAMIC MODEL FOR HAPTEN-ANTIBODY INTERACTION(S)

The conformational state of an antibody molecule following interaction with antigens was reviewed by Metzger.[5] It is quite obvious that no concensus could be reached from the evidence presented. It has, therefore, been considered desirable to explore the potential of the thermodynamic changes accompanying hapten (or antigen)-antibody interaction as indicators of possible conformational changes occurring in the formation of hapten-antibody complexes. Such studies must be carried out over a wide range of temperatures since the manifestation of conformational changes in protein interactions cannot be obviously described

either at one temperature or within a narrow range of temperatures. The importance of temperature in protein reactions, particularly those common in water solutions, has been extensively reviewed elsewhere[6] and is beyond the scope of this review. In order to relate the probable thermodynamic changes to the accompanying conformational changes in protein-ligand interactions, it is essential to investigate the physicochemical factors which contribute to and are therefore responsible for maintaining the conformational stability of proteins. These factors have also been extensively reviewed elsewhere[6] in terms of hydrogen bonds, hydrophobic bonding, coulombic interactions, etc. The difficulty lies in quantitating these factors and determining the nature of the factor(s) that dominate a particular thermodynamic situation.

By application of the concepts put forward by Lumry and Biltonen[6] it might be possible to evaluate the above parameters by rationalizing a possible thermodynamic model for the interaction of an antibody molecule with a univalent hapten. Now, let us consider an antibody combining site as consisting of appropriate amino acid side chains structured to a conformational state representing a hydrophobic locus containing one or more hydrophobic bonds (Figure 1).

Solvent molecules such as water are considered to be in an ordered structure in the native antibody molecule (structured water). On combination with the hapten in this hydrophobic locus, the solvent molecules may be assumed to be pushed out of the way, i.e., removed from the ordered solvent structure and transferred to the bulk solvent. Since there are less amino acid side chains in contact with the structured water in the bound state, the hydrophobicity of the combining site would naturally be expected to increase. The presence of hydrophobic loci in the antibody combining site has been amply demonstrated.[7,8]

A. Heat Capacity

In comparison with an unbound antibody molecule which has a higher heat capacity due to the solvent molecules being more structured, the bound antibody molecule upon interaction with hapten results in a less ordered solvent structure and, therefore, a lower heat capacity. Similarly, a change in heat capacity could be expected to occur owing to the disruption of bound water stabilizing the native conformation of the antibody combining site. However, an important point to bear in mind is that the overall magnitude of the change in heat capacity at constant pressure (ΔC_p) of an antibody molecule on combination with the hapten at various temperatures could either be positive or negative depending on its conformation in the bound state, i.e., unfolded vs. refolded.

B. Enthalpy

From a simplistic point of view, the change in enthalpy of binding may be explained by the following relationship:

$$\Delta H^\circ_{total} = \Delta H^\circ_{binding} + \Delta H^\circ_{solvent}$$

where ΔH°_{total} is the experimentally measured or calculated variable, and $\Delta H^\circ_{solvent}$ corresponds to the energy required to break up the structured solvent molecules in the native antibody molecule and would be expected to become smaller in absolute magnitude with increasing temperatures. $\Delta H^\circ_{binding}$, on the other hand, should stay essentially constant.

C. Entropy

The change in entropy in hapten-antibody binding may be explained in the following relationship:

$$\Delta S^\circ_{total} = \Delta S^\circ_{association} + \Delta S^\circ_{solvent} + \Delta^\circ_{internal}$$

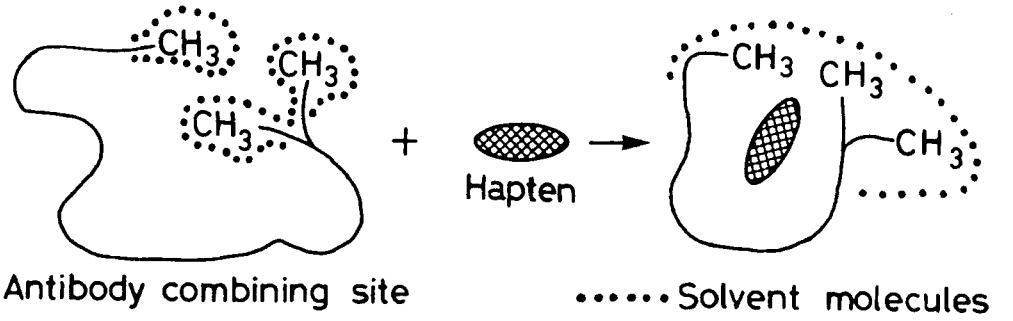


FIGURE 1. A hypothetical diagrammatic representation of hapten-antibody complex formation involving hydrophobic interaction(s).

where ΔS°_{total} (ΔS°_T) is the experimentally determined value. $\Delta S^\circ_{association}$ represents the standard entropy change associated with actual binding of hapten with the antibody combining site. As a result of binding, there would be a change from a "disordered" to an "ordered" state and $\Delta S^\circ_{association}$ should be a negative value.

$\Delta S^\circ_{solvent}$ is the standard entropy change associated with the reorganization of solvent molecules in, and possibly around, the antibody combining site upon binding with the hapten. This process results in the transfer of structured solvent molecules in the antibody combining site to the bulk solvent. Because this is an "order to disorder" process, $\Delta S^\circ_{solvent}$ should be a positive value.

$\Delta S^\circ_{internal}$ is the standard entropy change associated with any restrictions on the internal rotations, vibrations, etc. occurring as a result of the binding of hapten and antibody. This seems likely to be a "disorder to order" state and $\Delta S^\circ_{internal}$ should therefore be a negative value.

Above-mentioned considerations of the hypothetical thermodynamic model are presented mainly as a means of rationalizing the observed trends in the changes in enthalpy (ΔH°) and entropy (ΔS°_T) accompanying the binding of hapten to the antibody combining site. Although the involvement of the hydrophobic bond in hapten-antibody binding was selected as an example presenting the hypothetical thermodynamic model, similar considerations may apply to some other types of noncovalent interactions participating in the formation of hapten-antibody complexes keeping in mind that the magnitude of their thermodynamic contribution would be proportional to their degree of participation in the hapten-antibody binding process.

III. MEASUREMENT OF THERMODYNAMIC PARAMETERS

A. Equilibrium Constant (or Affinity)

The interaction of antibody and hapten (or antigen) at equilibrium may be expressed in the following way:

$$Ab + H \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ab\text{-}H$$

where Ab = free antibody; H = free hapten; Ab-H = the antibody-hapten complex; k_a = the association constant; and k_d = the dissociation constant. Applying the law of mass action to this interaction:

$$k_a [Ab] [H] = k_d [Ab\text{-}H] \quad (1)$$

Thus the equilibrium constant (or affinity) may be calculated:

$$\frac{k_a}{k_d} = K = \frac{[Ab\text{-}H]}{[Ab\ H]}$$

The application of the law of mass action in the derivation of the above thermodynamic parameter has been excellently reviewed by Day.[9] The concept of multiple equilibria describing the interaction of proteins with small molecules[10,11] has also been considered to be valid for the formation of hapten-antibody complexes.

The multiple equilibria for the binding of hapten, H, by a multivalent antibody, Ab, with n combining sites may be represented by the following reactions:

$$\begin{aligned} Ab + H &\rightleftharpoons AbH \\ AbH + H &\rightleftharpoons AbH_2 \\ AbH_{i-1} + H &\rightleftharpoons AbH_i \\ AbH_{n-1} + H &\rightleftharpoons AbH_n \end{aligned}$$

AbH_i is a species in which i molecules of H have been bound to Ab, the maximum value of i being n. The equilibrium constants for these reactions are

$$\begin{aligned} k_1 &= [AbH]/[Ab]\ [H] \\ k_2 &= [AbH_2]/[AbH]\ H] \\ k_i &= [AbH_i]/[AbH_{i-1}]\ [H] \\ k_n &= [AbH_n]/[AbH_{n-1}]\ [H] \end{aligned} \qquad (2)$$

where k_i is the equilibrium constant for the formation of the complex, AbH_i.

If AbH_i is assumed to denote molar concentration and not activity of the species, then:

$$\text{the total moles of bound H} = [Ab] \sum_{i=1}^{n} i(k_1,k_2 \ldots . k_i)\ [H]^i$$

$$\text{the total moles of protein} = [Ab] + Ab \sum_{i=1}^{n} (k_1,k_2 \ldots . k_i)\ [H]^i \qquad (3)$$

$$\frac{\text{total moles bound H}}{\text{total moles Ab}} = \frac{\sum_{i=1}^{n} i(k_1,k_2 \ldots . k_i)\ [H]^i}{1 + \sum_{i=1}^{n} (k_1,k_2 \ldots . k_i)\ [H]^i} = r$$

This results in a relatively simple expression provided the following assumptions are made:

1. The protein molecules involved in binding do not appreciably interact with each other.
2. All binding sites on the protein molecule are identical and act independently of one another.
3. The protein molecule does not affect the activity of the ligand except by binding.
4. Most importantly, the law of mass action is obeyed.

If each site on the antibody molecule is independent of each other, and each has the same intrinsic affinity or association constant, K, then the relationship between the equilibrium constants, $k_1,k_2 \ldots\ldots k_n$, can be described by the following relationship:

$$k_i = \frac{45 - i + 1}{i} K \tag{4}$$

where $n - i + 1/i$ is the statistical factor; k_i is the equilibrium constant for the formation of the complex, AbHi; K is the intrinsic affinity constant for the general reaction in which a representative antibody site Abs, binds a hapten molecule H, Abs + H $\rightleftharpoons$ AbsH.

By substitution of Equation 4 into 3 and application of the binomial theorem, the following expression may be obtained:

$$r = \frac{[H]\, nK \{1 + K [H]\}^{n-1}}{1 + \{K [H]\}^{n-1}} \tag{5}$$

or

$$r = \frac{nK [H]}{1 + K [H]} \tag{6}$$

where r = moles hapten bound per mole antibody; H = free hapten concentration (denoted frequently by the alphabet letter "C"); n = antibody valence; and K = equilibrium constant or affinity.

1. Experimental Methods for Determination of Bound and Free Hapten

A variety of experimental methods that permit the quantitation of free and bound hapten are now available in the literature (Table 1), the most commonly used methods being equilibrium dialysis and fluorescence quenching. However, because of the adequate experimental and theoretical considerations given to most of the methods listed in Table 1 elsewhere,[23,30,31] and in a recent monograph entitled *Antibody Affinity: Thermodynamic and Biological Significance* (Steward and Steensgaard, in press), the only method described below is the spectrophotometric method.[19,20]

A hapten-antibody system with novel spectral properties was reported by Wofsy.[24] The hapten 2-(2,4-dinitrophenyl azo)-1-naphthol 3,6-disulfonic acid, disodium salt (DNPNS) was found to undergo a marked and easily visible spectral change on specific binding to anti-DNP antibody at pH values near neutrality. It was suggested that the pK of the naphtholic OH group of DNPNS (Figure 2) was shifted to a higher value upon binding to the antibody and that the spectral change observed accompanying this antibody reaction was, therefore, primarily associated with the simultaneous protonation of the naphtholate ion group. Similar results have also been reported for another dye-hapten, 1-hydroxy-4(2,4-dinitrophenyl azo)-2, 5-naphthalene disulfonate (IN-2,5,S-4DNP)[19] and a solvato-chromic dye, 4-(3-aminophenyl)-2,6-diphenylpyridinium-N-(4-Hydroxyphenyl)betaine (ADHB).[21] However, it must be pointed out that an essential prerequisite for the use of this method is that the haptens used must undergo significant changes in absorption properties upon binding by specific antibodies.

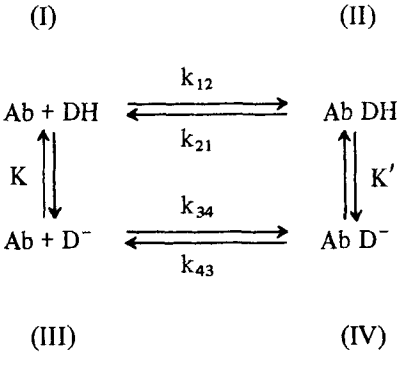
The absorption properties of DNPNS, a typical dye-hapten[20] are shown in Figure 3. On adding DNPNS to an equivalent or excess amount of anti-DNP antibody at pH 7 to 8, a striking change in the color of the hapten from blue to pink occurs instantly, no such spectral change being observed on its addition to the normal immunoglobulin, or antibodies to an heterologous hapten or serum albumin. At acid pH, the OH form of the free dye shows small shifts of the absorption maximum from 460 to 474 nm on binding to albumin and to 486 nm on binding to specific antibody (Figure 3). At pH 7.4, the free dye is largely in the naphtholate ion form (or O^- form) with an absorption maximum at 590 nm. Whereas on binding to albumin at this pH the maximum is shifted to 608 nm, on binding to anti-DNP

Table 1
METHODS FOR THE MEASUREMENT OF THE AFFINITY OF ANTIBODY-ANTIGEN REACTION

Method	Principle antigens used	Ref.
Equilibrium dialysis	Haptens, dialyzable antigens	19
Fluorescence quenching	Haptens and antigens with specific fluorescence properties	13
Fluorescent enhancement		14
Fluorescence polarization	Haptens, proteins	15
Ammonium sulfate globulin precipiation	Haptens, antigens soluble in 50% saturated ammonium sulfate	16
Dextran-coated charcoal	Haptens	17
Antiglobulin precipitation	Haptens, proteins carbohydrates	18
Spectral method	Dye-haptens, solvatochromic dyes	19 20 21
Equilibrium molecular sieving	Haptens, proteins carbohydrates	22

antibody the spectrum becomes very similar to that of antibody-bound hapten at acid pH, i.e., 486 nm. It, therefore, appears that on binding to albumin at pH 7.4, DNPNS remains predominantly in the O^- form, whereas on binding to specific antibody, the naphtholate ion becomes protonated. At pH 8.45, a secondary absorption peak with a maximum at 610 nm becomes discernible.

The following reaction mechanism has been proposed for the interaction of dye-haptens (IN-2,5S-4DNP) or DNPNS and anti-DNP antibody (Froese and Sehon, 1975).



where DH represents the hapten as the naphthol derivative; D^- the corresponding naptholate form; and K and K′ the acid dissociation constants. According to this scheme, the dye-haptens are preferentially bound in the naphthol form by the anti-DNP antibody and the binding is pH dependent. The equilibrium constant K for the reaction between states I and II can be calculated at any pH according to the following equation:[20]

$$K = \frac{1 + K/[H^+]}{1 + K'/[H^+]} Q$$

where

$$Q = \frac{[AbD]_t}{[Ab]\,[D]_t}$$

and the subscript t denotes total concentration at any pH.

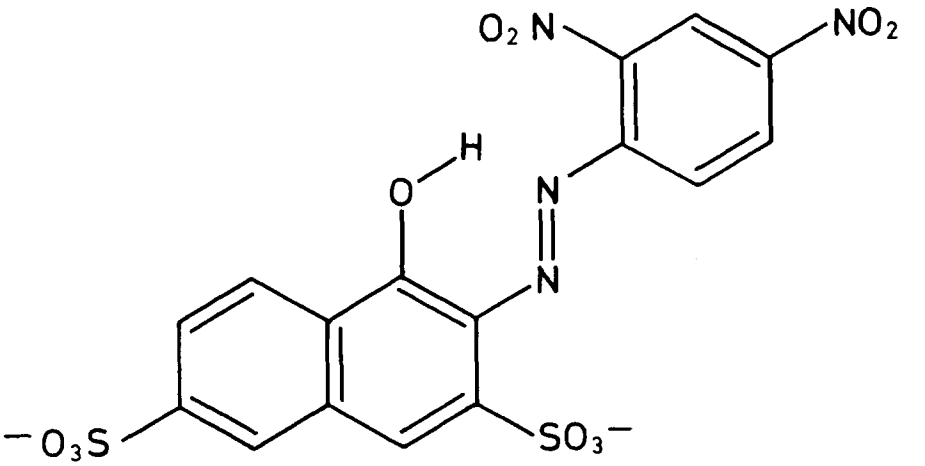


FIGURE 2. The hapten 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonate (DNPNS).

Because of the spectral shifts associated with the binding of the dye-haptens to specific antibodies, the extinction coefficients of the free and bound forms of the dye-haptens at a given pH may be calculated by their incremental addition to the solvent in the absence and presence of specific antibody (Figure 4). It can be seen that the optical density at 590 nm is a linear function of the added DNPNS,[20]for at least 65% of the theoretical number of antibody sites in the solution. It has been concluded that in this linear region essentially all the added hapten is bound to antibody with a constant and characteristic extinction coefficient. The upward curvature exhibited by the optical density at 590 nm upon further addition of DNPNS is considered to be due to the presence of increasing but small amounts of free DNPNS in equilibrium with antibody. While the bound DNPNS is predominantly in the OH form at pH 7.4 (Table 2), it is clear that the extinction coefficients represent the total bound DNPNS, i.e., the sum of contributions from the OH and O^- forms of the bound hapten.

The binding data can be calculated from measurements made at one wavelength if the total hapten concentration is precisely determined. However, because of the difficulty in estimating the latter precisely, particularly when titrations are performed in small volumes, it is important to make absorption measurements at two wavelengths, thereby obviating the requirement of knowing the total amount of hapten added. The concentration of the hapten in the bound (b) and the free (C) forms can then be calculated by the following equations:

$$b = \frac{\epsilon_{f1} A_2 - \epsilon_{f2} A1}{\epsilon_{f1} \epsilon_{b2} - \epsilon_{f2} \epsilon_{b1}}$$

$$C = \frac{\epsilon_{b2} A1 - \epsilon_{b1} A2}{\epsilon_{f1} \epsilon_{b2} - \epsilon_{f2} \epsilon_{b1}}$$

where ϵ and A denote extinction coefficient and absorbancy, respectively, and subscripts f and b refer to free and bound forms of the dye-hapten. The subscripts 1 and 2 denote the two wavelengths at which ϵ and A are determined.

2. Representation of Binding Data

Having determined the concentration of free and bound ligand (hapten), the intrinsic equilibrium or association constant or affinity (K) can be calculated by plotting the data either according to Scatchard,[25] Langmuir,[25a] and Sips[26] (Sips distribution function), or

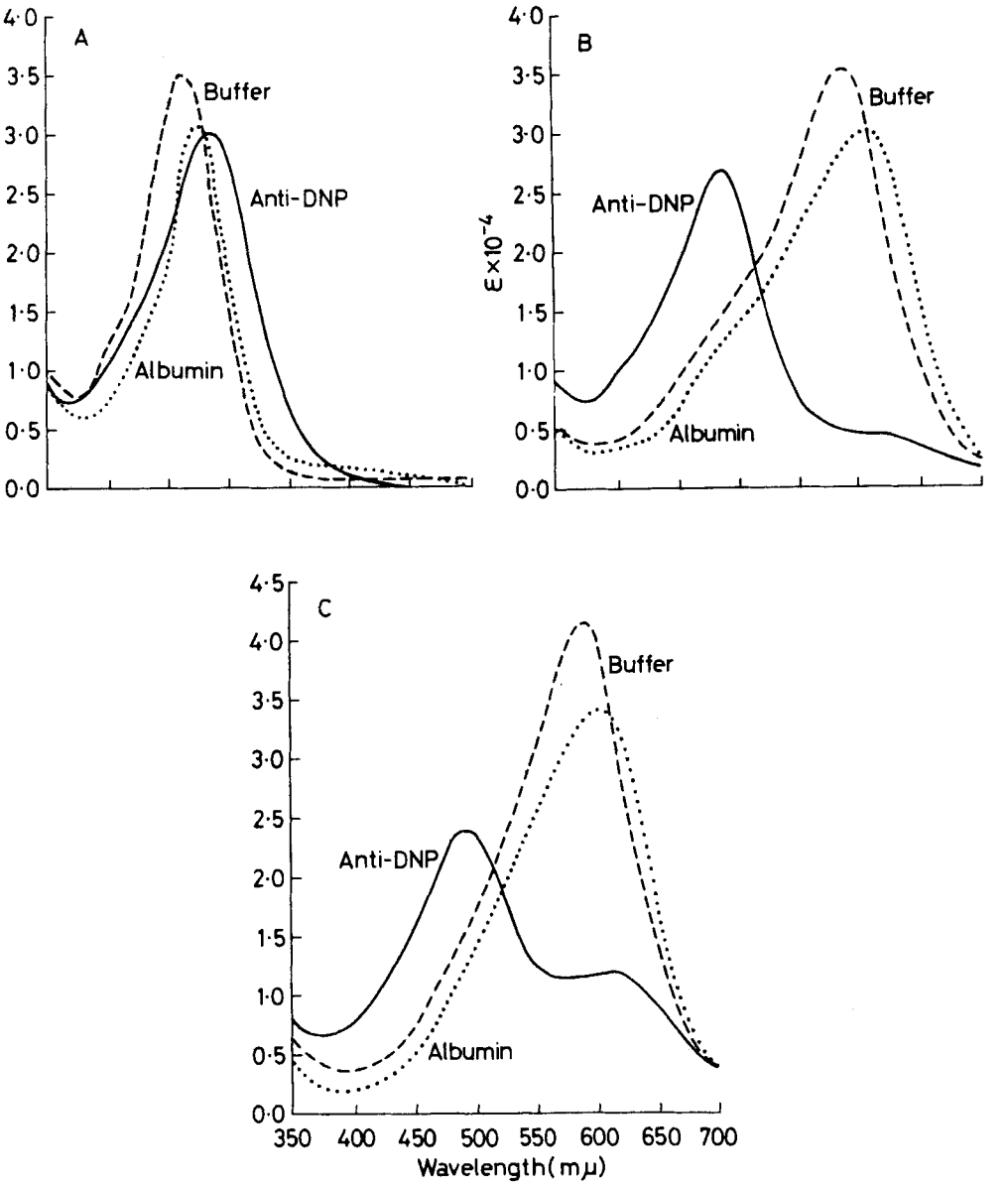


FIGURE 3. Spectra of DNPNS in the presence of anti-DNP antibody (6.1×10^{-5} *M*), of rabbit serum albumin (1.7×10^{-4} *M*), and of buffer alone. (A) In pH 4.7 buffer containing 0.01 *M* Na acetate and 0.17 *M* NaCl. (B) In pH 7.40 Na phosphate buffer. (C) In pH 8.45 Na borate buffer. In (A) and (B) and DNPNS concentration was 1.02×10^{-5} *M*, and in (C) 1.06×10^{-5} *M*. (From Metzger, H., Wofsy, L., and Singer, S. J., *Arch. Biochem. Biophys.*, 103, 206, 1963. With permission.)

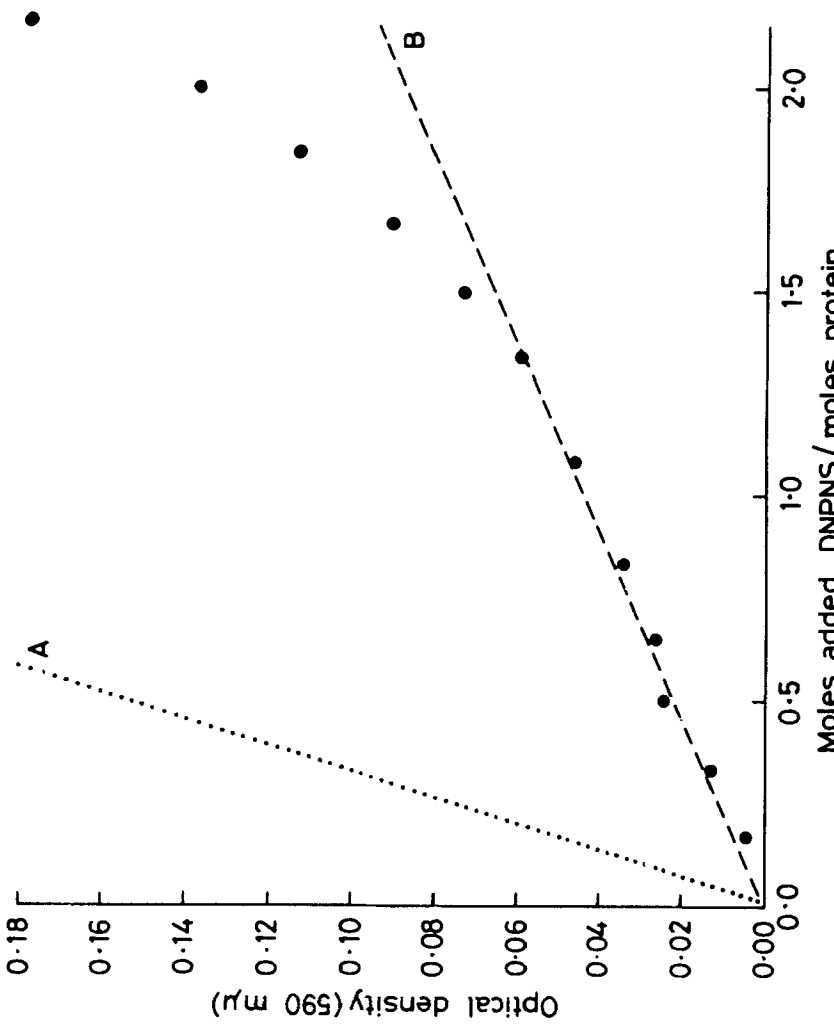


FIGURE 4. Spectrophotometric titration of the binding of DNPNS to anti-DNP antibody (9.7×10^{-5} *M*) in phosphate buffer, pH 7.40, at 25°C. The dotted line, A, represents the optical density of free DNPNS in buffer alone. The dashed line, B, yields the extinction coefficient at 590 nm of the antibody-bound DNPNS. (From Metzger, H., Wofsy, L., and Singer, S. J., *Arch. Biochem. Biophys.*, 103, 206, 1963. With permission.)

Table 2
SPECTRAL PROPERTIES OF DNPNS[20]

pH	nm	× 10^{-4} Free hapten	× 10^{-4} Bound hapten
4.7	460	3.49	2.41
	486	2.72	3.10
7.4	486	1.51	2.66
	590	3.58	0.398
8.45	486	1.51	2.40
	590	4.10	1.15

representing it as a log Q′ plot.[27] Since the former three methods of calculating affinity have been discussed in several publications and reviews,[28-31] they will be considered only in brief.

The log Q′ plot which permits one to determine the total affinity constant (K_t) of heterogeneous hapten-antibody interactions representing the sum of the weighted affinities of antibody subpopulations will be discussed separately (see Section III.A.3).

Rearrangement of Equation 6 yields:

$$\frac{r}{H} = -rK + nK \tag{7}$$

which is known as the Scatchard equation. A plot of r/H vs. r over a range of free hapten concentrations permits one to calculate antibody affinity (K) and the antibody valence (n) to be extrapolated (Figure 5).

Antibody affinity may also be calculated using the Langmuir plot (Figure 6) as shown below. From Equation 1:

$$K = \frac{[Ab - H]}{[Ab]\,[H]} = \frac{b}{[Abs - b]\,C} \tag{8}$$

where Abs = total concentration of antibody sites; b = bound hapten; and C = free hapten.

$$\begin{aligned} &K\,Abs\,C - KbC = b \quad \text{or} \\ &K\,Abs\,C - b(1 + KC) \quad \text{or} \\ &\frac{1}{b} = \frac{1 + KC}{K\,Abs\,C} \quad \text{or} \\ &\frac{1}{b} = \frac{1}{K\,Abs\,C} + \frac{1}{Abs} \end{aligned} \tag{9}$$

For ideal hapten (or antigen)-antibody binding, both the Scatchard and Langmuir equations should yield linear plots. However, when studying antibodies raised against haptens in conventional animals, the plots generally deviate from linearity. The reason for this curvature has been well documented to be due to the existence of heterogeneity of antibody affinities within an antibody population. In such cases an assumption has been made that the distribution of affinities can be described in terms of a Gaussian error function.[32,33] With this assumption, an average intrinsic association constant (K_o) can be calculated when half of the available antibody binding sites are occupied. Therefore, consider a point where

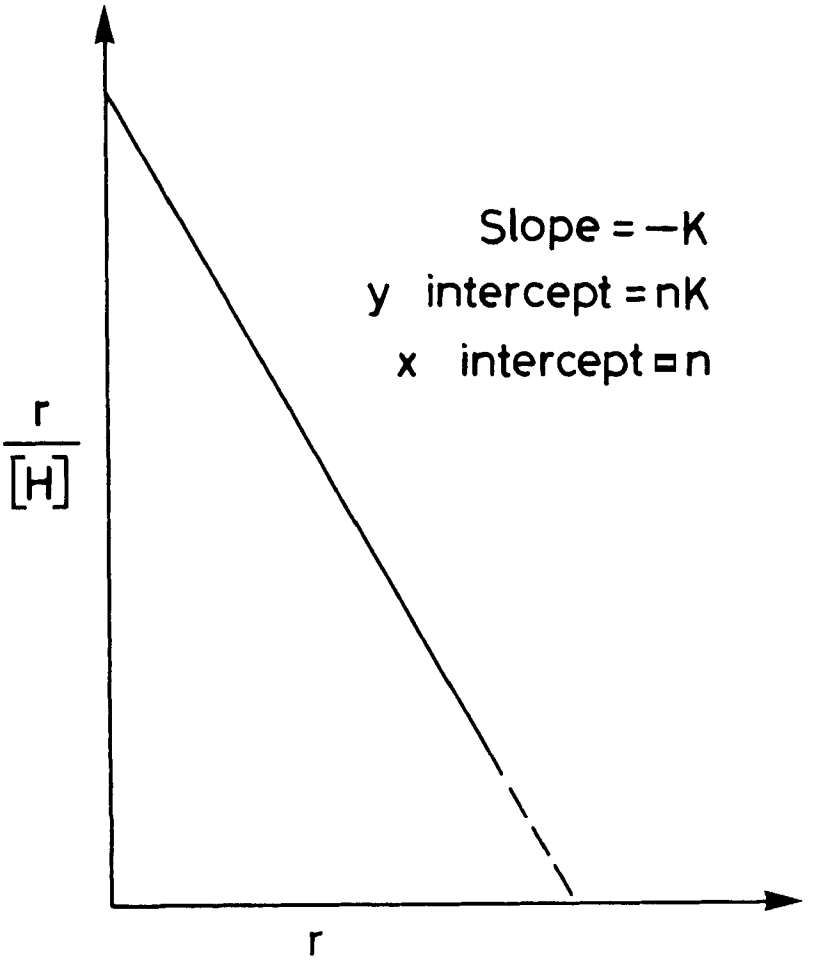


FIGURE 5. Diagrammatic representation of Scatchard plot for the interaction of homogeneous antibody with hapten.

$$b = \frac{Abs}{2} \qquad \text{(Figure 6)}$$

then

$$\frac{2}{Abs} = \frac{1}{KAbsC} + \frac{1}{Abs}$$

Hence,

$$K = \frac{1}{C} \text{ when } b = \frac{Abs}{2} \qquad (10)$$

Binding data can also be analyzed by using a Sipsian distribution function.[26] Nisonoff and Pressman[28] developed an equation relating r and C designed to reflect the heterogeneity of the antibody population. On integration the resulting binding equation can be written as

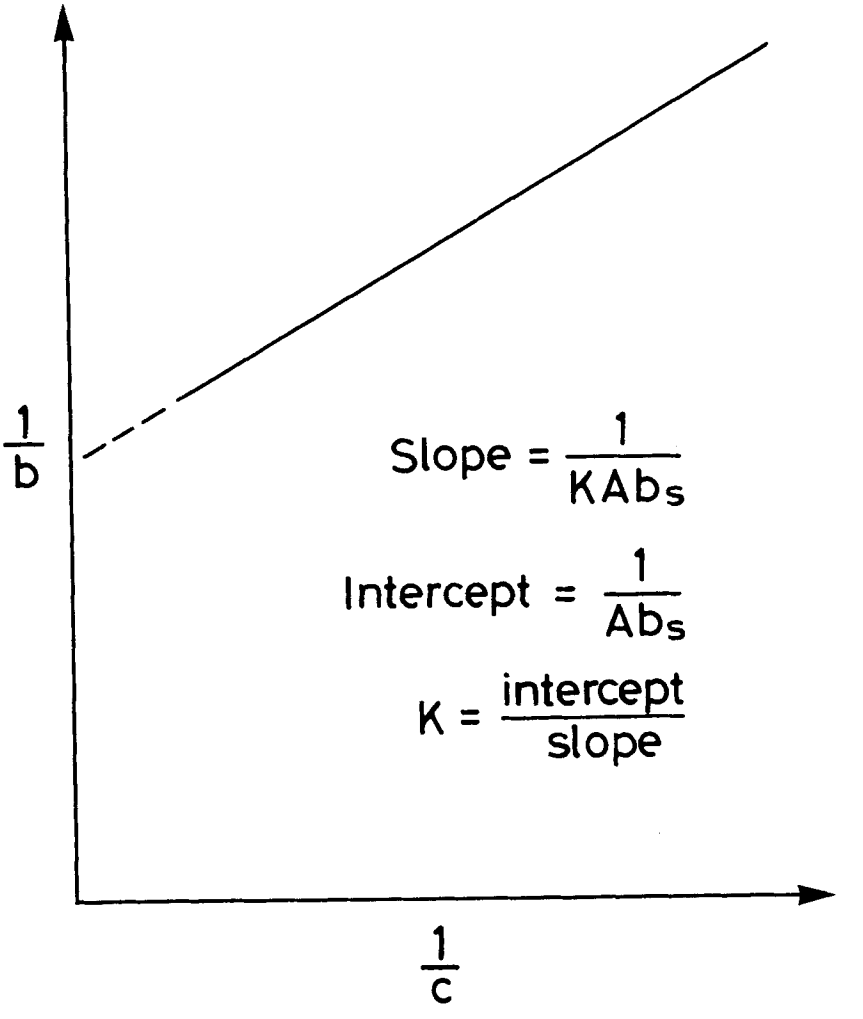


FIGURE 6. Diagrammatic representation of Langmuir plot for the interaction of homogeneous antibody with hapten.

$$r/n = \frac{(K_o C)}{1 + (K_o C)} \tag{11}$$

If r is expressed in fraction of antibody sites occupied n = 1:

$$\begin{aligned} \frac{1}{r} &= \frac{1}{(K^{\circ}C)^{\alpha}} + 1 \quad \text{or} \\ \frac{(K^{\circ}C)^{\alpha}}{r} &= 1 + (K^{\circ}C)^{\alpha} \quad \text{or} \\ (K^{\circ}C)^{\alpha} &= r + (K^{\circ}C)^{\alpha} r \quad \text{or} \\ (K^{\circ}C)^{\alpha} &= \frac{r}{1 - r} \quad \text{or} \\ \log\left(\frac{r}{1 - r}\right) &= \alpha \log C + \alpha \log K_o \end{aligned} \tag{12}$$

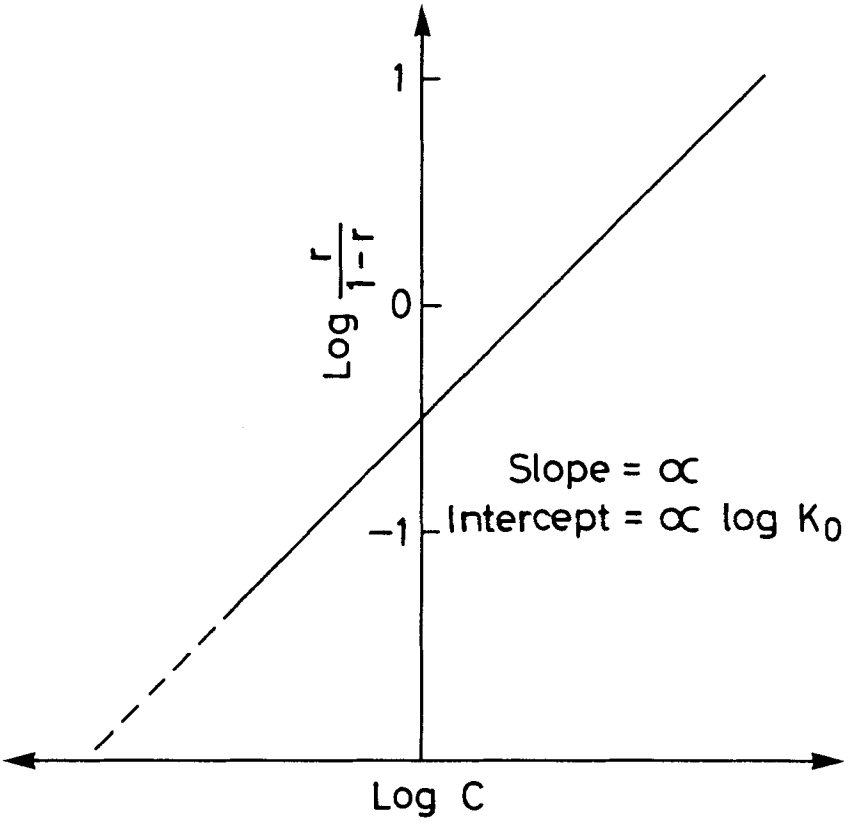


FIGURE 7. Diagrammatic representation of Sips plot for the interaction of homogeneous antibody with hapten (α = 1.0).

where α denotes the heterogeneity index. Binding data plotted according to Equation 12 are known as the Sips plot (Figure 7).

3. Total Affinity Constant (K_t)

The use of the average intrinsic association constant (K_o) for hapten-antibody interactions was based on the assumption that the free energy for the combination of ligands with antibody molecules followed Gaussian distribution.[32,33] However, it was clearly demonstrated by Werblin and Siskind[34] employing Sipsian distribution function that at periods other than very early after immunization, various subpopulations of antibody molecules were not symmetrically distributed about the mean with regard to affinity, but were skewed towards the higher affinity end of the distribution. Therefore, it was obvious that the affinity of hapten-antibody interaction cannot be legitimately represented by K_o. These investigators suggested the calculation of the affinity constant by the use of the binding data obtained when the initial 0.3 to 33% of the total binding sites was occupied, because the Sip's plot of the binding data in this range did not deviate significantly from a straight line. However, this method has a limitation in that it does not take into account the concentration of different antibody subpopulations with apparently different affinities.

Mukkur et al.[27] have reported the derivation of the total affinity constant, K_t, which represents the sum of weighted affinities of component subpopulations in an antibody preparation. Assuming the absence of inter- and intramolecular interactions between the binding sites of a heterogeneous antibody preparation composed of a series of m subpopulations:

$$R = \frac{K_1C}{1 + K_1C} \cdot N_1 + \frac{K_2C}{1 + K_2C} \cdot N_2 + \ldots + \frac{K_iC}{1 + K_iC} \cdot N_i + \ldots + \frac{K_mC}{1 + K_mC} \cdot N_m \quad (13)$$

where K_i = classical equilibrium constant for the ith antibody subpopulation in liters/mole; N_i = concentration of antibody sites in moles/liter with an equilibrium constant, K_i; m = number of subpopulation; R = equilibrium concentration of antibody-bound sites in moles/litre (these were determined by multiplying the concentration of antibody with r, the moles of hapten bound per mole of antibody as determined from the binding experiment); and C = equilibrium concentration of free hapten in moles/litre. Now let us define a function Q′ by the following relation:[35]

$$Q' = \frac{R}{(N - R)C}$$

where N = the total concentration of antibody binding sites in the reaction system in moles/litre, and determined by multiplying the concentration of antibody with n, the number of combining sites/mole of antibody as determined from the Scatchard plot.

Substitution of Equation 13 for R into the above relationship gives the desired expression:

$$Q' = \frac{\frac{K_1}{1 + K_1C} \cdot N_1 + \frac{K_2}{1 + K_2C} \cdot N_2 + \ldots + \frac{K_i}{1 + K_iC} \cdot N_i + \ldots + \frac{K_m}{1 + K_mC} \cdot N_m}{\frac{N_1}{1 + K_1C} + \frac{N_2}{1 + K_2C} + \ldots + \frac{N_i}{1 + K_iC} + \ldots + \frac{N_m}{1 + K_mC}} \quad (14)$$

For an antibody sample containing only two subpopulations (m = 2), Equation 14 can be rearranged as follows:

$$Q' = \frac{\frac{K_1N_1 + K_2N_2}{N} + K_1K_2C}{1 + \frac{N_1K_2 + N_2K_1}{N} \cdot C}$$

The limiting value of Q′ as C approaches zero is given by the relation:

$$\lim_{C \to 0} Q' = K_t = \frac{K_1N_1 + K_2N_2}{N} \quad (15)$$

Similarly for an antibody sample containing m subpopulations, the limiting value of Q′ as C approaches zero can be readily shown to be

$$\lim_{C \to 0} Q' = K_t \frac{K_1N_1 + K_2N_2 + \ldots + K_iN_i + \ldots + K_mN_m}{N} \quad (16)$$

or

$$K_t = \sum_{i=1}^{m} \frac{N_iK_i}{N}$$

It is obvious then that the K_t represents the sum of weighted affinities of m antibody subpopulations in the total reaction system. A plot of log Q′ as a function of R *or* r would give a curve with a negative slope for a heterogeneous binding system, whereas a horizontal straight will be obtained for a homogeneous antibody preparation. The antilogarithm of the extrapolated value of log Q′ when R *or* r approaches zero (which is equivalent to when C approaches zero) would represent the total affinity constant, K_t (Figure 8). Since the K_t represents the sum of weighted affinities of different antibody subpopulations, whose number is almost impossible to estimate,[36] it could perhaps be even better described as the number average association constant. However, it must be pointed out that the binding data at low ligand concentrations are very important in order to perform such extrapolations accurately. Therefore, the total affinity constant, K_t, would appear to be a more legitimate representation of the affinities of all the subpopulations in an antibody preparation without either prior assumption about their number of possible subpopulations or their relative concentration.

The total affinity constant (K_t) has been experimentally validated and a comparison of the computer-calculated and graphically extrapolated values for various antibody preparations showed an excellent agreement (Table 3). The computational treatment utilized an iterative procedure to obtain least-square estimates of K and N, according to Marquardt.[37]

4. Concentration Dependence of Binding Due to Nonspecific Interaction of Hapten with Antibody

In any investigation involving determination of thermodynamic parameters, it is not only imperative to make corrections for contributions due to nonspecific interaction of hapten with antibody molecules, but it is also important that the immunoglobulin used for affecting these corrections is of the same species as that employed for raising antihapten antibodies and not of heterologous origin. Johnston et al.[38] reported that the interaction of MOPC 315 myeloma protein with ϵ-DNP-L-lysine was concentration dependent in that an increase in antibody concentration led to a decrease in the equilibrium constant, suggesting the existence of intermolecular negative cooperativity. However, the investigators considered it safe to ignore these interactions on the grounds that such interactions could not be accompanied by a large enthalpy change since in thermal experiments the protein solution underwent a fourfold dilution in the calorimeter from 10 to 2.5 mg/mℓ without significant enthalpy change.

On the other hand, whereas some investigators studying the interaction of haptens with specific antihapten antibodies reported that the average intrinsic association constant, K_o, of the subunits was not significantly different from their parent intact antibodies,[39-41] implying the absence of intramolecular and possibly intermolecular interactions, others reported a slight reduction of affinity constants,[42,43] thus suggesting the opposite. The above interpretation was based on the assumption that if inter- and intramolecular interactions were present, reduction of the intact antibody molecule to its subunits might result in either negative or positive cooperativity.

We have studied the question of intermolecular interactions by examining the concentration dependence of the total affinity constant, K_t, for the interaction of ϵ-DNP-L-lysine and bovine anti-DNP IgG1.[44] It was discovered that as the concentration of anti-DNP IgG1 was increased, the K_t decreased, although the number of combining sites per mole of IgG1 increased (Table 4). These results were unexpected, since, if at all, an increase in binding (most likely attributable to nonspecific binding) should have been observed. These data, therefore, suggested the possible presence of intermolecular interactions as reported previously by Johnston et al.[38] in the mouse system. However, when nonspecific binding of ϵ-DNP-L-lysine by increasing concentrations of normal colostral IgG1 was measured using one hapten concentration, a negative slope was observed (Figure 9). However, the amount of nonspecifically bound hapten at a given antibody concentration increased with increasing hapten concentration until near saturation. When the binding data for each concentration of anti-DNP IgG1

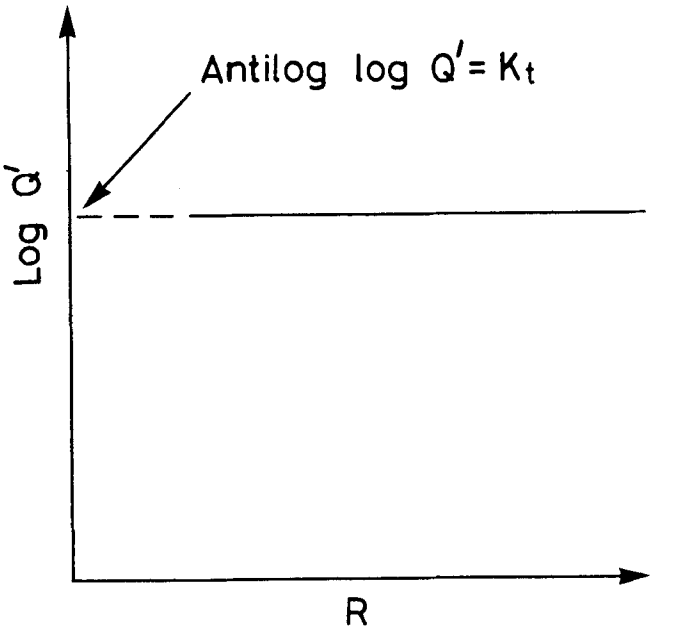


FIGURE 8. Diagrammatic representation of log Q′ plot for the interaction of homogeneous antibody with hapten.

Table 3
COMPARISON OF INTERPOLATED AND COMPUTER-CALCULATED TOTAL AFFINITY CONSTANTS (K_t) EXPRESSED AS LITERS PER MOLE FOR VARIOUS ANTIBODY PREPARATIONS[27]

	Rabbit anti-DNP IgG		Rabbit anti-DNP IgG[a]		Bovine anti-DNP IgM	
Subpopulation assumption (m)	Interpolated	Computer calculated	Interpolated	Computer calculated	Interpolated	Computer calculated
	× 10^{-6}	× 10^{-6}	× 10^{-6}	× 10^{-6}	× 10^{-5}	× 10^{-5}
2	1.3 ± 0.3	1.52	2.2 ± 0.2	2.22	3.9 ± 0.2	3.63
3		1.54		2.22		3.65
4		1.54		2.24		3.65
5		1.54		2.23		3.65

[a] Data obtained through the courtesy of Dr. Eisen (McGuigan, J. E. and Eisen, H. N., in *Methods in Immunology and Immunochemistry*, Vol. 3, Williams, A. C. and Chase, M. W., Eds., Academic Press, New York, 1971).

were corrected for nonspecific binding, the curves were completely superimposable in the Scatchard plot (not shown) as well as in the log Q′ plot (Figure 10, A and B). These results strongly suggested that the specific binding of ϵ-DNP-L-lysine by anti-DNP IgG1 and hence the K_t were independent of the concentration of antibody used in the binding experiments, and indicated the absence of intermolecular interactions. Further binding experiments conducted with the Fab′ and Fc′ fragments of nonspecific IgG1 revealed that the negative cooperativity effect was localized in the Fc′ fragment. That the observed negative cooperativity was possibly a result of a conformational change(s) in the antibody molecule was obtained on sedimentation analysis of bovine IgG1 in the presence of excess ϵ-DNP-L-lysine (Table 5) when the formation of polymers up to 16.9S was observed.

Table 4
NUMBER OF COMBINING SITES AND TOTAL AFFINITY CONSTANT (K_t) OF BOVINE ANTI-DNP IgG1 AT INCREASING CONCENTRATIONS BEFORE AND AFTER CORRECTION FOR NONSPECIFIC BINDING[44]

Concentration of antibody (%)	Before correction for nonspecific binding		After correction for nonspecific binding	
	K_t (M^{-1})	n	K_t (M^{-1})	n
0.35	2.23×10^4	1.95	0.58×10^4	1.95
0.50	0.85×10^4	2.3	0.58×10^4	2.0
0.70	0.53×10^4	2.3	0.58×10^4	2.0

In order to find out if the negative linear slope was a general characteristic of nonspecific binding of ε-DNP-L-lysine to normal immunoglobulins from various species, two other systems were tested. While normal human IgG yielded similar results, bovine IgG2 gave a positive, linear slope (figures not shown), which again is suggestive of significant structural and conformational differences between some inter- and intraspecies immunoglobulins. It would be of interest to determine if the mechanism of negative cooperativity reported in the mouse system by Johnston et al.[38] is similar to that reported for bovine IgG1, i.e., unrelated to the specific binding of hapten to the antibody combining site.

Another feature that must not escape attention is the self-associating property of the antibody molecule under investigation. Such factors as the ionic strength and the pH of the buffer system used could prove quite important in as much as the self-association properties of the antibody molecule are concerned. For example, Mukkur and Smith[45] have found that the bovine serum and colostral IgG1 undergo self-association in 0.32 *M* NaCl to 0.01 *M* Tris-HCl, pH 8.0 to form dimers, but not in 0.16 *M* NaCl to 0.01 *M* sodium phosphate, pH 6.0 with association constants of $5.3 \pm 3.5 \times 10^4\ M^{-1}$ and $1.6 \pm 0.69 \times 10^3\ M^{-1}$, respectively. Although we have not quantitated the exact contributions that the above-mentioned properties of an antibody molecule could make to the overall thermodynamic measurements, it is obvious that these sorts of corrections are necessary in order to obtain unequivocal data.

B. Free Energy Change

The standard free energy change, ΔG° for hapten-antibody interaction (Equation 1) may be obtained from the relation:

$$\Delta G^\circ = -RT \ln K \tag{17}$$

where R is the gas constant; T the absolute temperature; and K the experimentally determined equilibrium constant. The value of ΔG° corresponds to the formation of 1 mol of Ab-H complex in which the initial activities of the reactants Ab and H and the final activity of product Ab-H complex are unity. Since in the case of haptens (or antigens) and antibodies, determinations of K are always made in dilute solutions, no distinction between the activities and molar concentrations has been considered necessary.

The ΔG for a reaction occurring at physiological concentrations (ΔG physiol) may be markedly different from ΔG°, since the concentrations of participating reactants may differ by orders of magnitude from those employed for direct experimental measurement. $\Delta G_{physiol}$ may be calculated as proposed by Klotz:[79]

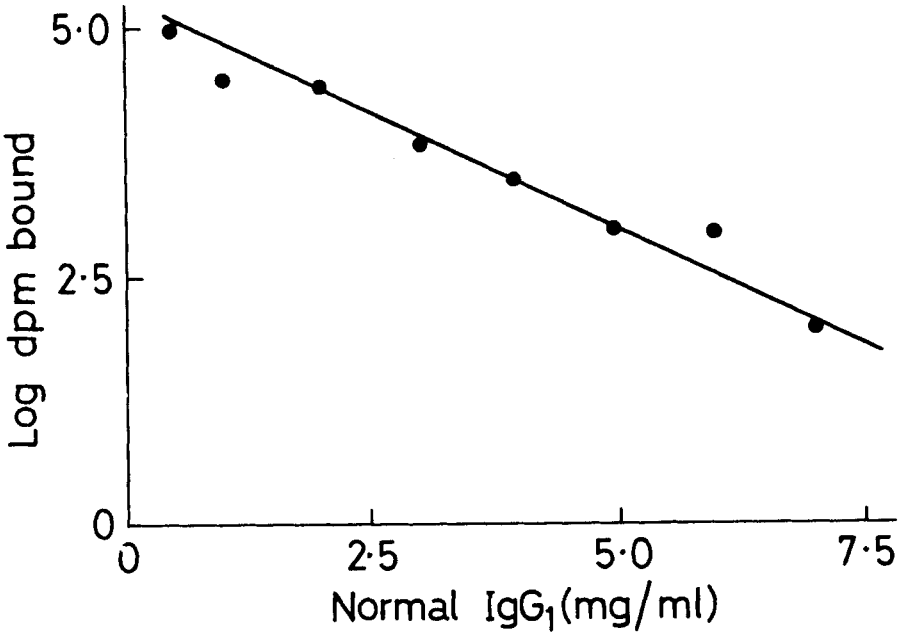


FIGURE 9. Demonstration of negative cooperativity for the interaction of ε-DNP-L-lysine with increasing concentrations of normal bovine colostral IgG. (From Mukkur, T. K. S. and Fang, W. D., *Anal. Biochem.*, 75, 183, 1976. With permission.)

$$\Delta G_{physiol} = \Delta G^\circ + RT \ln K_{physiol} \quad \text{or}$$

$$\Delta G_{physiol} = -RT \ln K + Rt \ln K_{physiol}$$

However, $\Delta G_{physiol}$ can only be obtained when physiological concentrations of the reactants and the products can be estimated.

The free energy change for the combination of hapten and antibody is composed of an enthalpic and an entropic term expressed as follows:

$$\Delta G = H - T\Delta S \tag{18}$$

In accordance with the condition $\Delta G < 0$ for a spontaneous reaction at constant temperature and pressure, two classes of spontaneous reactions can be distinguished. In the first case the reaction is enthalpy driven, i.e., the term ΔH is predominant. In the second case the reaction, nonexothermic or even endothermic, is entropy driven. Alternatively, the same reaction could be enthalpy driven at one temperature and entropy driven at another, a phenomenon referred to as enthalpy-entropy compensation.[4,6] The discrimination is of particular interest in terms of understanding the nature of forces implicated in the formation of hapten-antibody complex.

C. Enthalpy Change

The enthalpic quantity ΔH is obtained directly by calorimetric measurement of the heat of reaction at constant pressure or indirectly determined from measurements of the temperature dependence of the equilibrium constant. Since dilute solutions of the reactants are generally employed, the experimental value of ΔH corresponds to that for an infinitely dilute solution and can be equated to ΔH°. Either a batch[2] or a flow[46] calorimeter can be used to measure the heat of binding (ΔH_o). The latter should be measured over a range of hapten

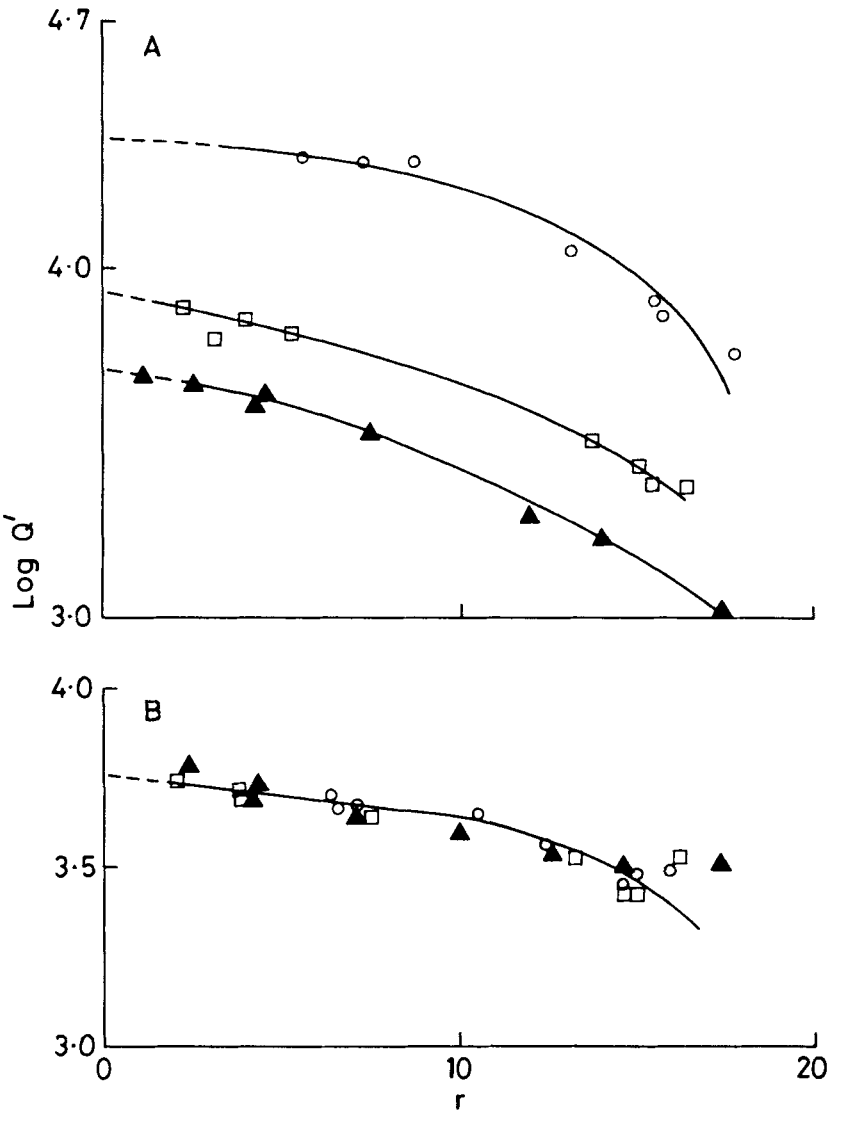


FIGURE 10. Log Q' plots for the interaction of ϵ-DNP-L-lysine with increasing concentrations of bovine colostral IgG_1. (A) The binding data are uncorrected for nonspecific binding. (B) The binding data are corrected for nonpecific binding. (From Mukkur, T. K. S. and Fang, W. D., *Anal. Biochem.*, 75, 183, 1976. With permission.)

concentrations, the hapten $[H_p]$ always being present in large excess. The heat is then extrapolated to infinite hapten concentration by a double-reciprocal plot of $1/\Delta H_b$ vs. $1/[H_p]$, the reciprocal of the y intercept yielding the binding enthalpy. The heat capacity (ΔCp) can then be calculated using the following relation:[38]

$$\Delta Cp = \frac{\Delta H_b(T_2) - \Delta H_b(T_1)}{(T_2 - T_1)} \tag{19}$$

where $\Delta H_b(T_1)$ and $\Delta H_b(T_2)$ are the heats of binding determined experimentally at two different temperatures, T_1 and T_2. For additional information on the use of calorimetry in biochemistry, an excellent review by Jolicoeur[47] is highly recommended. Again, as with the

Table 5
SEDIMENTATION ANALYSIS OF NORMAL BOVINE COLOSTRAL IgG1 BEFORE AND AFTER THE ADDITION OF EXCESS ε-DNP-L-LYSINE[44]

	$S_{20,w}$ of IgG1 and various components	
Concentration of IgG1 (%)	Before hapten addition (S)	After hapten addition (S)
0.35	6.6	6.9
0.50	6.5	7.6, 15.5
0.70	6.4	8.0, 16.9

equilibrium constants, the importance of making appropriate corrections due to nonspecific interaction of hapten with antibody cannot be overemphasized.

Most often the value of heat of reaction itself has been derived from measurements of equilibrium constant K at two different temperatures. According to the van't Hoff relation:

$$\frac{d\ln K}{dT} = \frac{\Delta H^\circ}{RT^2} \tag{20}$$

and over small ranges:

$$\ln \frac{K_1}{K_2} = \frac{\Delta H^\circ}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{21}$$

where ΔH° is the sum of the enthalpies of the products minus the enthalpies of the reactants when each is at its reference state. However, the above equation is valid only if a plot of lnK vs. 1/T yields a linear relationship. We have found that in the case of anti-DNP antibody if the temperatures chosen for the determination of equilibrium constants fall within a narrow range, the van't Hoff plots are generally linear. However, if the determinations are carried out over a wide range of temperatures, the van't Hoff plots are nonlinear[49-51] (also Szewczwk and Mukkur, unpublished) (Figures 11, A to E). We have derived an equation that can be used to describe nonlinear variations of association constants with temperature.[49]

At equilibrium as in Equation 17:

$$\Delta G^\circ = RT\ln K_t$$

where K_t is the total affinity constant;[27] R the gas constant; and T the absolute temperature.

When dealing with reactions over a temperature range, $\Delta C^\circ p$, the standard heat capacity change, and ΔH°, the standard enthalpy change may be represented by equations of the algebraic type:

$$\Delta C^\circ p = \Delta a \tag{22}$$

$$\Delta H^\circ = \Delta b + \Delta aT \tag{23}$$

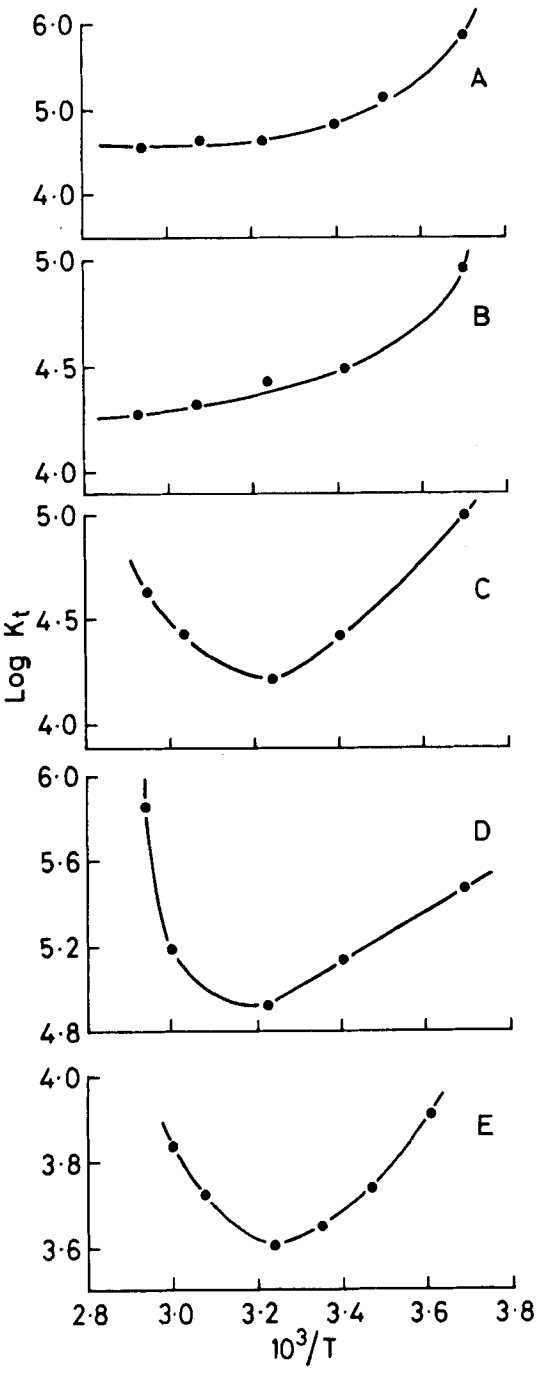


FIGURE 11. van't Hoff plots for the interaction of ϵ-DNP-L-lysine with anti-DNP antibodies.[49-51] (A) Bovine colostral IgG1. (B) $F(ab')_2$ fragment of bovine colostral IgG1. (C) Fab' fragment of bovine colostral IgG1. (D) Rabbit IgG. (E) Bovine colostral IgG2.

where a is the algebraic sum of all the values of a, etc. Substituting this last expression into the differentiated equation for free energy as a function of temperature:

$$\left(\frac{\delta(\Delta G/T)}{\delta(1/T_p)} = \Delta H\right)$$

and integrating:

$$\Delta G^\circ = \Delta b - \Delta aT\ln T + CT \tag{24}$$

where C is the integration constant.

Substitution of Equations 23 and 24 into the equation for free energy change as a function of temperature ($-\Delta S = \Delta G - \Delta H/T$) yields:

$$\Delta S^\circ_T = \Delta a + \Delta a\ln T - C \tag{25}$$

where ΔS°_T is the standard entropy change. Substitution of Equation 17 into Equation 24 yields:

$$\ln K_t = \frac{\Delta a}{R}\ln T - \frac{\Delta b}{R} \times \frac{1}{T} - \frac{C}{R} \tag{26}$$

The graphic representation of the van't Hoff plot consists of a plot of $\ln K_t$ or $2.303 \log K_t$ against 1/T. The nonlinear variation of association constants with temperature could be described by Equation 26. The three unknown constants may be calculated by simultaneous equations or by a least-squares computer procedure as described previously[27] and employed to calculate ΔC°_p, ΔH°, and ΔS°_T.

D. Entropy Change

The standard entropy change (ΔS°_T) may be calculated from experimental determinations of ΔG° and ΔH° by application of the equation for the free energy change as a function of temperature ($-\Delta S = \Delta G - \Delta H/_T$). The quantity ΔS°_T is an essential parameter for the interpretation of the mechanism of hapten (or antigen)-antibody reactions in terms of the formation and rupture of different types of bonds by hapten (or antigen) and antibody with water and between each other. Entropy changes may also reflect transconformations of regions of the antibody molecule distant from the combining site. For such interpretations, it may be desirable to eliminate that part of ΔS°_T which does not depend on structural modifications, but is strictly related to the mixing of the molecules with the solvent. This part of the change of entropy has been called the entropy of mixing and its elimination can be readily achieved using the concept of unitary entropy.[52] By taking into account the number of states in which a molecule may occur inside a solvent and by considering that the molecule could have been in the place occupied by any molecule of the solvent, the entropy of mixing for 1 g molecule per litre of water (55.6 mol) is $R \ln 55.6 = 7.98$ cal/deg^{-1} for a sufficiently dilute solution. The molar entropy of a substance corrected by 7.98 cal/deg^{-1}, is called the unitary entropy and can be obtained by the relation:

$$\Delta S_u = \Delta S^\circ + R \ln 55.6 = \Delta S^\circ + 7.98 \tag{27}$$

In accordance with Equation 27, the unitary free energy ΔG_u, dependent only on structural modifications, defined as $\Delta H - T\,\Delta S_u$, is given by:

$$\Delta G_u = \Delta G^\circ - 7.98\,T \tag{28}$$

For greater details on the utility of unitary entropy and unitary free energy as the basis for discussing different types of bonding and association reactions, a review dealing with the analysis of protein denaturation by Kauzmann[53] is highly recommended.

IV. RESULTS OF THERMODYNAMIC STUDIES

A. Hapten (Simple)-Antibody Interaction(s)

In initial studies, Barisas et al.[54] reported calorimetric determinations of the enthalpy of binding of various dinitrophenyl (DNP) haptens to rabbit anti-DNP antibodies. The results of the thermal titration of intact antibody indicated that for at least the tighter binding part of the population of antibody sites, the binding enthalpy was quite large, i.e., -22.8 ± 0.4 Kcal/mol of sites. At saturation of antibody sites by hapten, the enthalpy decrease averaged over all sites was -15.2 ± 0.3 Kcal/mol. On the other hand, the fluorometric titration of antibodies led to an enthalpy value based on the van't Hoff equation of -11 Kcal/mol. It was suggested that the enthalpy change observed was much larger than the spread in the standard free energy changes indicated by the Sips heterogeneity index calculated using the fluorometric titration data. Because of the similarity in the shape of the thermal titration curves obtained with univalent Fab fragments and intact antibodies, it was concluded that the enthalpy variation observed was due to site heterogeneity rather than to site interaction in the bivalent antibody molecule. A year later, Barisas et al.[55] repeated their initial work[54] and also investigated the thermodynamics for the interaction of the 2,4,6-trinitrophenyl group (TNP) to homologous and heterologous rabbit IgG. The binding of ϵ-N-2,4,6-TNP-L-lysine to rabbit anti-TNP antibody at 25°C was found to be accompanied by a decrease of 21.7 ± 0.4 Kcal/mol of ligand bound and a decrease in the apparent heat capacity of -155 cal/deg^{-1}/mol^{-1}. However, in these experiments, an examination of the thermal titration curves both for the interaction of ϵ-N-2,4,6-TNP-L-lysine and ϵ-N-2,4-DNP-L-lysine to rabbit anti-TNP and anti-DNP antibodies, respectively, indicated no heterogeneity in the binding enthalpies. It was, therefore, concluded that the earlier conclusion[54] suggesting the existence of marked heterogeneity of binding enthalpy may have been incorrect. Therefore, it was considered possible that perhaps the Sips distibution function used for calculating the average association constant (K_o) did not give a proper representation of the heterogeneity in the binding data. The latter assumption has been well documented by Werblin and Siskind.[34]

Because of the conflicting data obtained above, Johnston et al.[38] repeated their experiments but used mouse myeloma proteins MOPC 315 and MOPC 460 instead, the latter having been demonstrated to be homogeneous in their binding to nitrophenyl ligands. In these experiments, the binding enthalpies, ΔH_b, obtained by direct flow calorimetry were reported to agree well with the van't Hoff values obtained from equilibium measurements made at 4 and 25°C. At 25°C, ΔH_b for systems involving polynitroaromatic ligands ranged from -12.1 to -20.2 Kcal/mol of ligand bound and was concluded as providing the driving force for ligand-protein interaction(s). The change in heat capacity (ΔC_p) which was calculated from the temperature variation of the calorimetrically determined ΔH_b was found to range from -110 to -300 cal/deg^{-1}/mol of ligand bound. From the calorimetric data on the ΔH_b for the association in water of tryptophan with DNP- and TNP-aminocaproic acid, it was suggested that charge transfer association of ligands with the tryptophan in the antibody combining site may contribute to the spectral and thermodynamic parameters of binding. In order to see whether the equilibrium measurements on protein 315 were sensitive to variation in protein concentration and to insure that it would be justifiable to combine the free energy values obtained by fluorescence quenching and equilibrium dialysis (0.064 mg/mℓ) with enthalpic measurements obtained calorimetrically (2.5 mg/mℓ), Johnston et al.[38] investigated the concentration dependence of the dissociation constant for the protein 315-DNP-lysine complex on protein concentration when a marked concentration dependence of the dissociation constant, presumably resulting from protein-protein interactions, was evident. The dissociation constant was found to increase by a factor of 10 as the protein concentration was raised from 0.04 to 10 mg/mℓ, whereas the valence remained constant (1.92 to 2.04

mol of hapten per mole of protein), the latter being in contrast to the findings reported previously (Underdown et al.[56]). However, no corrections due to concentration dependence were made in the thermal titration experiments based on a number of assumptions, most significant of which was that since the protein solution underwent a fourfold dilution in the calorimeter, from 10 to 2.5 mg/mℓ with no significant enthalpy change, the protein-protein interaction (if the latter was indeed responsible for the observed variation in the affinity constants) was not accompanied by a large enthalpy change. Unfortunately, no further studies on the concentration dependence of proteins 315-DNP-lysine interaction on lines similar to those reported for the interaction of bovine anti-DNP IgG1 and ϵ-DNP-L-lysine[44] have been forthcoming. If the concentration dependence observed was due to the nonspecific interaction of the hapten with the Fc region of protein 315 as has been reported to be the case with bovine anti-DNP IgG1, then equilibrium and calorimetric measurements carried out with the Fab subunits of protein 315, preferably over a wider range of temperatures than that employed previously,[38] could provide relatively more comprehensive information. A further difficulty arises from the fact that either no corrections to the enthalpy of binding due to the nonspecific binding of hapten to intact antibody have apparently been made[38] or the immunoglobulin used for such corrections has been of heterologous origin.[54]

Halsey and Biltonen[2] have studied the thermodynamics for the interaction of ϵ-DNP-1-lysine with heterogeneous rabbit IgG fractions with different affinities by the use of a calorimeter. An average ΔH of -13.9 Kcal/mol was measured. Although there was no correlation between the enthalpy and free energy changes, a statistically significant correlation was observed between the free energy changes and unitary entropy changes which varied from -5.1 to -10.6 cal per mole deg. (Table 6). Since hydrophobic interactions are thought to be involved in the formation of hapten-antibody complexes, one would have predicted a positive entropy contribution. Based on their observation of a negative heat-capacity change for the transfer of DNP-1-lysine from water to ethanol, these authors attempted rationalization by suggesting that perhaps this large negative entropy contribution was associated with other aspects of hapten-antibody interaction. One drawback in this particular investigation was that the immunoglobulin employed for affecting corrections due to nonspecific binding of hapten to antibody was of heterologous origin, i.e., bovine IgG. Whereas similar exothermic enthalpy changes were also obtained for the interaction of 2,4-DNP-L-lysine with goat anti-DNP antibodies[38a] (Table 6), the enthalpy change with guinea pig antibodies was much less exothermic (-8.7 Kcal/mol). The latter value was the same for two guinea pig antibodies that differed in affinity by almost two orders of magnitude. These results were interpreted to indicate that the heterogeneity in affinity was primarily the result of differences in the entropy of interaction. In the case of guinea pig antibodies, positive entropy changes were associated with the binding reaction. These findings suggested that the hydrophobic interactions were probably more important in guinea pig than in rabbit and goat antibody, the entropy change being negative in the latter interaction(s). Archer and Krakauer[48] determined the thermodynamic parameters characterizing the binding of dinitrophenyl-glycine (DNP-glycine) and dinitrophenyl-methoxypoly-ethylene glycol (DNP-MP) with equine anti-DNP IgG(T) antibodies fractionated according to affinity. The techniques employed included fluorescence quenching at ambient temperatures (22 to 25°C) and flow calorimetry presumably also performed at or near ambient temperature. The binding enthalpies and entropies were in all cases large and negative in the ranges of -14 to -17 Kcal/mol and -18 to -25 entropy units (eu), respectively. The binding enthalpy under conditions of excess sites and excess hapten was fitted to straight lines and extrapolated to the equivalence point because in the case of DNP-MP, interaction with nonspecific immunoglobulin was apparent. From limited determinations, it was concluded that the differences in binding affinity of the same hapten to different antibodies were due to differences in enthalpy of binding which were assumed to reflect differences in the specific interaction

Table 6
COMPARISONS OF THE HIGH AND LOW AFFINITY FRACTIONS OF RABBIT AND GUINEA PIG ANTI-DNP ANTIBODY

	Kcal/mol	
	ΔG	ΔH
Rabbit antibody[2]		
High affinity (fractions I and II)	−9.72	−13.85 ± 0.59
Low affinity (fractions III, IV, and V)	−8.46	−13.93 ± 0.48
Guinea pig antibody[38a]		
Anti-DNP-I	−12.1	−8.7
Anti-DNP-II	−9.45	−8.8

of the hapten with the binding sites. The latter conclusion, while supportive of the calorimetric results reported by Johnston et al.,[38] is in direct contrast to those of Halsey and Biltonen[2,38a] who found that populations of rabbit and guinea pig antibodies differing in average affinity by 1.3 and 2.65 Kcal/mol, respectively, had indistinguishable binding enthalpies (Table 6). Johnston et al.[59] have reexamined the question of the distribution of binding enthalpies in conventionally raised antibody populations against the 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP) groups by the use of three methods which avoided physical fractionation of the antibody samples into affinity classes: (1) direct calorimetry; (2) temperature dependence of the Sips heterogeneity index as measured by equilibrium dialysis; and (3) temperature-jump van't Hoff measurements. The first method showed that the binding enthalpies ΔH of the tightest binding sites were indistinguishable from those of the average sites. For the second and third methods, Johnston et al. (1969) defined an affinity binding enthalpy correlation parameter f which was *zero* if all the affinity groups had the same ΔH and which is *one* if variations in ΔG° among groups were attributable to those in ΔH. Values of f greater than *one* were considered to indicate differences in ΔH among groups larger than the corresponding differences in ΔG°, these enthalpic differences being partially compensated by entropic factors which acted to reduce the free energy differences between groups. The second method yielded results which were consistent with f = o for both the anti-DNP and anti-TNP antibody preparations. The third method dealt with the temperature dependence of $d\theta/dT$ of the total extent of saturation θ of a heterogeneous system obtained by determining the specific incease in fluorescence resulting from the dissociation of hapten-antibody complex using an apparatus especially designed for efficient collection of emitted fluorescence radiation. Since a plot d^{θ}/dT vs. θ revealed values of f that fell between 0 and 1.5, it was concluded that the distribution of binding constants in the anti-DNP and anti-TNP systems was at least partly caused by enthalpic heterogeneity. As pointed out earlier, these results differed from those of Halsey and Biltonen[2,38a] in which case the hapten-binding enthalpies were relatively constant, and the variations among binding entropies were considered to account for the observed affinity differences. Clearly, the calorimetric experiments have yielded quite diverse results and need to be performed not only over a wider range of temperatures, but also employing appropriate controls for affecting corrections due to non-specific binding of hapten to antibody before any definite conclusions could be reached.

The binding enthalpy values for the interaction of Lac dye with rabbit antilactose antibody fractions,[60] calculated from binding data obtained by equilibrium dialysis, ranged from −9.5 to 11.5 Kcal/mol of hapten bound. This narrow range of values was interpreted to suggest that the antibody-ligand interaction utilized almost the full capacity of the lactosyl moeity

for hydrogen bond formation in an antibody site. It was, therefore, concluded that the ΔH term was primarily due to multiple hydrogen bonding and that there was no significant change in the heat capacity associated with the formation of hapten-antibody complex. The contribution of hydrophobic bonding in this system was not assigned any significant role. These results essentially confirmed those reported earlier.[33] Velick et al.[13] evaluated the thermodynamics for the interaction of rabbit anti-DNP antibody with ϵ-DNP-l-lysine over a wide range of temperatures (4 to 60°C) employing fluorescence quenching for the determination of equilibrium constants. The van't Hoff plots yielded a straight line and the standard enthalpy change (ΔH^b) for complex formation was found to be −8.6 Kcal/mol. The unitary entropy change (ΔS_u) was calculated to be 17 eu/mol. Velick et al.[13] concluded that enthalpy and not entropy factors were dominant in strong binding. Since values for ΔH^b and ΔS_u were not calculated for each temperature, the relative contribution of enthalpy and entropy to the binding process at each temperature could not be evaluated.

In our thermodynamic investigations, for the interaction of ϵ-DNP-l-lysine with rabbit anti-DNP IgG,[49] bovine anti-DNP IgG1[49] and its fragments, $F(ab')_2$ (Szewczwk and Mukkur, unpublished) and Fab'[50] and bovine anti-DNP IgG1,[51] we employed the total affinity constant (K_t) in the construction of van't Hoff plots because K_t represents the sum of weighted affinities of an antibody population and therefore takes into account not only the affinities, but also the concentrations of the subpopulations in an antibody sample (Equation 16). In our studies which were conducted over a wide range of temperatures (−3 to +67°C), nonlinear van't Hoff plots were obtained (Figures 11, A to E.) This forbade the use of the classical van't Hoff equation[61] (Mahan, 1963) as a result of which appropriate equations were therefore, derived (see Section III.C). The unitary free energy and the unitary entropy changes were then calculated. The extent of curvatures of the van't Hoff plots was found to be indicative of large positive heat-capacity changes, ΔC_p° (Table 7) which were indicative of the unfolding of an antibody combining site as a result of combination with the hapten. The discrepancy the van't Hoff profiles reported earlier[13,38] and those reported by us[49-51] may be due to the fact that fluorescence quenching tends to measure antibody with relatively high affinity in comparison with equilibrium dialysis which has been used to measure antibodies with affinity as low as $10^3\ M^{-1}$.[62] On plotting various thermodynamic functions for the interaction of anti-DNP antibodies and their fragments (Table 8) vs. temperature, enthalpy-entropy compensation was revealed (Figures 12, A to E). The unfavorable entropy factor at low temperatures appeared to be compensated by the negative enthalpy factor resulting in a favorable free energy change for each of the anti-DNP antibody preparations. Therefore, it would appear that the enthalpy factor was a primary contributor to the driving force for the formation of hapten-antibody complex at lower temperatures as suggested previously,[54,55,58,60] but the entropy factor assumed greater importance at increased temperatures, thus permitting one to rationalize a mechanism for hapten-antibody interaction(s) in terms of enthalpy-entropy compensation effect which has previously been used to explain various enzymatic-catalytic reactions.[63] The predominance of enthalpy factor at low temperatures could be rationalized by suggesting the predominance of hydrogen bonding possibly formed for each bound hapten.[60,64] However, it does not rule out the participation of hydrophobic interactions, the latter being expected to be predominant if the entropy contributions are positive instead of unfavorable negative unitary entropy changes.[54,55,60] That hydrophobic bonding possibly participates in the formation of hapten-antibody complexes in spite of a negative heat-capacity change has already been suggested[2] because of the observed negative heat-capacity change for the transfer of DNP-lysine from water to ethanol.

Our experiments,[49-51] however, revealed a positive heat capacity change (ΔC_p°) for the interaction of ϵ-DNP-l-lysine and anti-DNP antibodies of rabbit and bovine origin (Table 7) which, according to Brandt,[65] indicates an increase in the number of strictly nonpolar side chains immersed in bulk water, thus suggesting an unfolding process having taken place.

Table 7
STANDARD HEAT-CAPACITY CHANGES (ΔC_p°) AND THE TEMPERATURE (°C) AT WHICH THE ENTHALPY FACTOR EQUALS THE ENTROPY FACTOR, BEFORE AND AFTER CORRECTION FOR THE ENTROPY OF MIXING, FOR THE INTERACTION OF ε-DNP-L-LYSINE WITH ANTI-DNP ANTIBODIES OF RABBIT AND BOVINE ORIGIN

Antibody preparation	ΔC_p°	Temperature (°C) at which the enthalpy factor equals the entropy factor (T_c)	
		Before correction	After correction
Rabbit anti-DNP IgG	+472.2	17.5	16
Bovine anti-DNP IgG1	+332.0	41.5	39
Bovine anti-DNP $F(ab)'_2$[a]	+118	37	35
Bovine anti-DNP Fab' of IgG1	+362.0	26	22
Bovine anti-DNP IgG2	+280.1	25	19.5

[a] Szewczuk, M. R. and Mukkur, T. K. S., unpublished results.

If one assumed that the negative values of ΔH_{total} at low temperatures were predominantly due to $\Delta H_{binding}$ of ε-DNP-l-lysine with specific antibody, the trend towards increasing positive values with increasing temperatures would be compensated by $\Delta H_{solvent}$, thus suggesting a possible mode for the participation of solvent-protein interactions in the formation of hapten-antibody complexes.

The entropy effects could also be rationalized in terms of a compensation mechanism. The observed trend in ΔS_u could be considered to represent a compensating effect(s) of $\Delta S_{internal}$ and $\Delta S_{solvent}$, the former being more important at lower and the latter at higher temperatures. If ΔS°_{total} (ΔS°_T) was corrected for the entropy of mixing to obtain ΔS_u, the unitary entropy change, and utilized for the compensation plot, enthalpy-entropy compensation was observed again, with the exception that the temperature at which the enthalpy factor equaled the entropy factor was slightly lowered (Table 7), thus suggesting an insignificant contribution of the entropy of mixing to the entropy due to specific hapten-antibody interaction(s). Further, the ΔS_u increased to positive values with increasing temperature (Table 8), thus suggesting a greater contribution of hydrophobic bonding in the formation of hapten-antibody complexes. Although the data on conformational changes accompanying hapten-antibody interaction(s) are not all unequivocal,[66-68] it is accepted that conformational changes do occur at least in antigen-antibody systems and thereby may contribute to the increase in entropy in addition to that due to the increase in solvent freedom resulting from the hydrophobic interaction.[69] It is interesting, then, that Epstein et al.,[57] who studied the interaction of haptens containing the phenylarsonic acid group with rabbit antiarsanilic antibody using a light scattering method at temperatures ranging from 15 to 37°C, reported a positive entropy change of 22 ± 9 eu/mol bonds at 25°C. These results were explained in terms of the liberation of a number of water molecules on combination of hapten with the antibody molecules.

The temperatures at which the enthalpy factor equaled the entropy factor (Tc) before and after the entropy of mixing are shown in Table 7. It was interesting to note that there was a statistically significant reduction in the Tc on proteolytic digestion of the intact bovine

Table 8
THERMODYNAMIC FUNCTIONS FOR THE INTERACTION OF ε-DNP-*l*-LYSINE, AND RABBIT AND BOVINE ANTI-DNP ANTIBODIES

Temperature (°C)	Rabbit anti-DNP IgG			Bovine anti-DNP IgG1			Bovine anti-DNP Fab' IgG1			Bovine anti-DNP IgG2		
	ΔG_u	ΔH°	ΔS_u	ΔG_u	ΔH°	ΔS_u	ΔG_u	ΔH°	ΔS_u	ΔGu	ΔH°	ΔS_u
−3	−9.27	−14.22	−18.34	−9.65	−18.20	−31.90	−8.72	−13.96	−19.38			
4										−7.06	−8.05	−3.64
12				−9.31	−13.29	−13.96						
15										−7.07	−4.97	+7.27
20	−9.83	−3.35	+20.32				−8.63	−5.27	+11.45			
22				−9.23	−9.97	−2.51						
25										−7.18	−2.17	+16.75
36							−8.95	+0.16	+29.46			
37	−9.87	+4.69	+46.99	−9.32	−0.50	+13.96				−7.49	+1.19	+27.89
52				−9.65	−0.02	+29.64						
57	−11.11	+14.15	+78.55				−9.81	+7.76	+53.27	−7.99	+5.40	+41.13
60										−8.32	+7.65	+47.94
67	−11.95	+18.88	+90.69	−10.19	+0.50	+44.62	−10.29	+11.38	+64.17			

Note: While ΔG_u and ΔH° are expressed in Kcal/mol, ΔS_u is expressed in entropy units (eu). The data presented in this table have been calculated from results published previously (References 49 to 51).

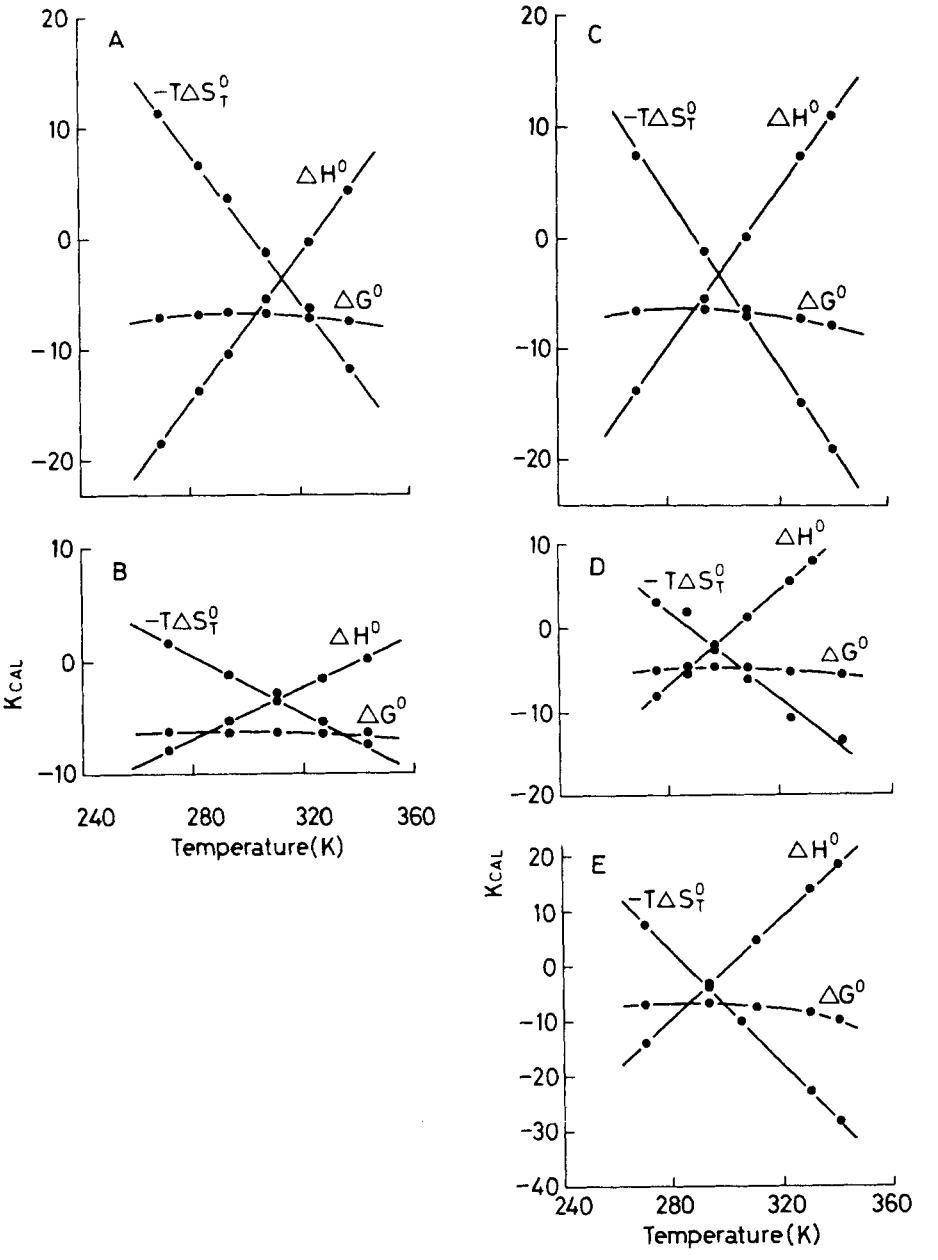


FIGURE 12. Compensation effect of thermodynamic parameters for ε-DNP-L-lysine binding by anti-DNP antibodies.[49-51] (A) Bovine colostral anti-DNP IgG1. (B) $F(ab)'_2$ fragment of bovine colostral IgG1. (C) Fab' fragment of bovine colostral IgG1. (D) Bovine colostral IgG2 (E) Rabbit IgG.

IgG1 to Fab' fragments. It is tempting to speculate that such a drastic reduction was due to the removal of the Fc region of IgG1 in light of the fact that the K_t for anti-DNP Fab' was approximately tenfold lower than that of the parent-intact anti-DNP IgG1. When ΔH° was plotted vs. ΔS_T° or ΔS_u in the form of a compensation plot, a straight-line relationship was observed in all cases, thus suggesting that a single kind of change in protein-solvent conformation (Lumry and Biltonen, 1969) had occurred owing to the formation of hapten-antibody complex. A typical compensation plot is shown in Figure 13.

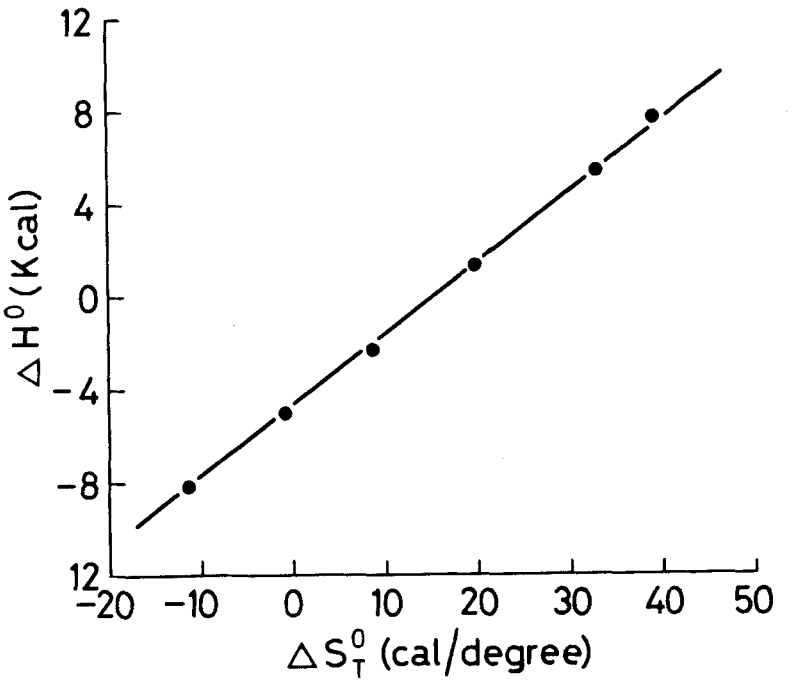


FIGURE 13. Compensation plot for the interaction of ϵ-DNP-L-lysine with bovine colostral anti-DNP IgG2. (From Mukkur, T. K. S., *Biochem. J.*, 172, 39, 1978. With permission.)

B. Hapten (Complex)-Antibody Interaction(s)

Whereas the thermodynamic information for the interaction of specific antibody with haptenic peptides is virtually nonexistent, that for interaction with oligosaccharides is scanty. Bhattacharjee et al.[70] studied the binding of antigalactan IgA (J539) and antidextran IgA (W3129) using a number of galactose- and glucose-containing ligands, respectively, at different temperatures and reported the enthalpies to be negative in both cases, representing the likely "removal" of solutes from solution upon binding to a protein. In the case of W3129, the enthalpies of binding were more favorable which offset the unfavorable entropies of W3129-ligand association. In the case of J539, the absolute values for the enthalpies were generally lower and the entropies were positive or slightly negative, thus indicating a larger favorable contribution of entropy. These investigators rationalized the above observations by suggesting that in the case of J539-galactose oligosaccharide interaction, the ligand made close contact with the protein "squeezing out" water of hydration (positive entropy effect), whereas in the case of W3129, the oligosaccharides are immobilized by binding three end residues to the protein, leaving the remainder of the ligand molecule out of contact reach with the protein, but still surrounded by structured water (negative entropy contribution). During their investigation of the binding site of dextran-specific homogeneous IgM (MOPC-104E), Schepers et al.[71] determined the enthalpy and entropy changes accompanying the interaction of MOPC-104E with native and derivitized oligosaccharides (alditols or danysylated oligosaccharides). Interaction of MOPC-104E with native tetrasaccharide revealed a negative ΔH (determined calorimetrically) and a slightly positive ΔS_u. Whereas similar results were obtained for the interaction of MOPC-104E with the corresponding alditol derivative, the dansylated derivatives yielded much higher binding entropy values, the highest ΔS_u observed being $+11.7$ eu for the dansyl tetrasaccharide as opposed to $+2.0$ eu for an alditol tetrasaccharide. Because of differences between their binding enthalpies ($\Delta H = -5.5$ and -7.4 Kcal/mol, respectively), it was suggested that interaction with

either the sulfohydrazine group or the dimethylaminonaphthyl ring became significant. It was also stressed that there was no immobilization of the fluorophore, implying that it formed no stable contact with the protein and exerted its entropic contribution by causing water molecules to be liberated from the binding site and/or from the hapten.

Although the above two groups of investigators[70,71] did not directly address themselves to the mechanistic aspects of oligosaccharide-antibody interaction(s), it seems highly likely that the thermodynamic mechanism for such interactions may also be described as enthalpy-entropy compensation.

C. Antigen-Antibody Interaction(s)

Thermodynamic information regarding the interaction of multivalent antigens with specific antibodies is scanty. The first such report to appear in the literature dealt with the interaction of bovine serum albumin with a crude preparation of antibovine serum albumin antibody.[1] The heat of reaction determined calorimetrically was negative, but the entropy change was calculated to be positive. Worobec et al.,[72] investigating the thermodynamics of angiotensin (I and II)-antibody interaction, reported that at 25°C the changes in the standard free energy, enthalpy, and entropy were, respectively, -8.4 to -13.1 Kcal/mol, -3.1 to -5.7 Kcal/mol, and 14.3 to 28.5 eu during the course of the immune response. The major contribution to the free energy was made by the entropic term which continued to increase during the course of the immune response. It was suggested that the energetics of this peptide-antibody interaction involved primarily hydrophobic interaction and, secondarily, van der Waals forces, coulombic forces, and hydrogen bonds in the resultant anhydrous environment.[73] Such reactions are essentially athermal, entropy driven, and accompanied by volume increases.[53,74] The above results essentially confirmed an earlier report describing the thermodynamic mechanism of the interaction of insulin and antiinsulin antibody[75] whereby the reaction was found to be predominantly entropy driven.

Halsey and Biltonen[2] during their investigation of the thermodynamics for the interaction of ε-DNP-L-lysine and rabbit anti-DNP antibody also determined the binding enthalpy (ΔH) for the interaction of rabbit anti-DNP antibody with dinitrophenylated human serum albumin (HSA-DNP) by the use of a calorimeter. The ΔH for the reaction was determined to be -10.1 Kcal/mol which was 3.8 Kcal/mol less exothermic than the ΔH for the hapten-binding reaction. The authors have suggested that this decrease could possibly be compensated by entropic contributions owing to enhanced functional affinity because of multivalence of antigen and antibody.[76]

van Oss et al.[77] have recently investigated the thermodynamics for the interaction of BSA with goat anti-BSA antibody employing precipitation in tubes at optimal ratios. The antigen-antibody reaction was found to be less exothermic at higher than at lower temperatures (ΔH became less negative at higher temperatures), whereas the positive entropy (ΔS) of the reaction increased with temperature (Table 9). It was, therefore, concluded that the phenomenon of enthalpy-entropy compensation reported for hapten-antibody interaction(s)[4] also holds true for the interaction of BSA with anti-BSA antibodies. These findings are highly significant in that the forces participating in the formation of specific antigen-antibody precipitates include not only those involved in primary binding, but also those involved in the formation of aggregates of antigen-antibody complexes (presumably coulombic and Van der Waals forces[78]) leading eventually to the formation of a visible precipitate.

Clearly the results presented above for the interaction of multivalent ligands and antibodies are preliminary and represent an area of research which needs further investigation.

V. CONCLUDING REMARKS

The thermodynamic data deduced from van't Hoff measurements clearly indicate that the mechanism for formation of hapten- and antigen-antibody complexes can fundamentally be

Table 9
THERMODYNAMIC DATA ON A BSA-GOAT ANTI-BSA SYSTEM (IgG FRACTION) OBTAINED BY PRECIPITATION IN TUBES AT OPTIMAL RATIO

	4°C	20°C	37°C
K (l/*M*)	11.16×10^7	5.03×10^7	3.34×10^7
Δ F(Kcal/*M*)	−10.2	−10.2	−10.7
Δ H(Kcal/*M*)	−7.6	−6.5	−4.6
Δ S(eu: cal/deg/mol)	+9.4	+12.9	+19.6

described as enthalpy-entropy compensation. The driving force for the formation of hapten-antibody complexes is enthalpy at lower temperatures and entropy at higher temperatures, their possible overall magnitude of contribution depending not only on the chemical nature of the ligand employed in the thermodynamic investigation, but also on the nature of the contact or closely proximal amino acids in the antibody combining site.

Data obtained by calorimetry, on the other hand, are still equivocal in that whereas one group of investigators is supportive of enthalpy as the primary driving force for the formation of hapten-antibody complexes and considers it to be at least partially responsible for the observed heterogeneity, another group has presented evidence to show that the heterogeneity of antibody affinity is primarily due to differences in the entropy of interaction. Further thermal titrations conducted over a wide range of temperatures and employing appropriate immunochemical controls may, therefore, be desirable to reach definitive conclusions. Future thermodynamic investigations should be carried out with particular emphasis on:

1. The thermodynamic mechanism(s) for the interaction of multivalent antigens with specific antibodies
2. The influence of antibody polyvalency on various thermodynamic functions
3. The relative quantitative contribution of various types of forces participating in the formation of hapten/antigen-antibody complexes

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