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Significant discrepancies between van't Hoff and calorimetric enthalpies

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ABSTRACT **In this paper we show that the usual assumption in studies of the temperature variation of equilibrium constants for equilibria of the form A + B ⇄ AB that a plot of ln K vs. $1/T$ (K = equilibrium constant, T = temperature in degrees kelvin) is a straight line with slope equal to $-\Delta H_{vH}/R$ (ΔH_{vH} = van't Hoff or apparent enthalpy, R = gas constant) is not valid in many cases. In all the cases considered here, ΔH_{vH} is temperature dependent and is significantly different from the true or calorimetrically measured enthalpy, and the respective values for ΔC_p are also significantly different.**

The ready availability of sensitive isothermal titration calorimeters makes possible the accurate determination of both enthalpies and equilibrium constants on the same sample for a wide variety of processes. In view of this situation, detailed comparisons of calorimetrically determined enthalpies of reaction (ΔH_{cal}) and enthalpies derived from equilibrium constants by means of the van't Hoff equation (ΔH_{vH}) can now be made. We have recently made such comparisons on a variety of systems, with quite unexpected results.

Titration calorimetric measurements are usually carried out over relatively narrow ranges of temperature such as 5–45°C. It is usually found in such cases that a plot of ln K vs. $1/T$, where K is the equilibrium constant for the reaction under study, appears quite linear, and linear least-squares analysis of the data leads to a correlation coefficient of 0.95 or higher, a value close enough to unity to be taken ordinarily as indicating linearity within experimental uncertainty. However, if one subjects the data to nonlinear least-squares analysis, employing the integrated form of the van't Hoff equation

$$\ln\frac{K}{K_o}=\frac{\Delta H_o - T_o\Delta C_p}{R}\left(\frac{1}{T_o}-\frac{1}{T}\right)+\frac{\Delta C_p}{R}\ln\frac{T}{T_o} \qquad [1]$$

with a nonvanishing but temperature-independent heat capacity change (ΔC_p), the curvature of the van't Hoff plot becomes evident. The importance and the generality of inclusion of the temperature variation of enthalpy in the van't Hoff equation have recently been emphasized by Weber (1). T_o in Eq. **1** is an arbitrarily selected reference temperature, K_o is the equilibrium constant, and ΔH_o is the van't Hoff enthalpy at that temperature. The three parameters varied to minimize the standard deviation of calculated values of ln K from those observed are ΔH_o, ln K_o, and ΔC_p. The assumption of a temperature-independent ΔC_p is generally valid over the limited temperature ranges involved.

The results obtained by this treatment of calorimetric titration data may be illustrated by studies we have recently made of the binding of cytidine 2′-monophosphate (2′-CMP) to ribonuclease A (RNase A) in 0.2 M potassium acetate buffer containing 0.2 M KCl at pH 5.5 with added 0.50 M sucrose. A reasonably good fit of the calculated to the observed binding constants was obtained, with ΔH_{vH} showing a 2-fold change between 15 and 40°C. It should be noted that linear least-squares analysis of ln K against $1/T$ gave an apparent temperature-independent ΔH_{vH} of −9.47 kcal·mol^{-1}, with a correlation coefficient of 0.979.

Table 1. Calorimetric titrations of RNase A with 2′-CMP at pH 5.5 in the presence of 0.50 M sucrose

t, °C	$K_B \times 10^{-5}$, M^{-1} Calculated	$K_B \times 10^{-5}$, M^{-1} Observed	ΔH_{vH}, kcal·mol^{-1}	ΔH_{cal}, kcal·mol^{-1}	$\Delta H_{cal}/\Delta H_{vH}$
15	1.344	1.410	−6.10	−8.82	1.45
15	1.344	1.240	−6.10	−8.97	1.47
16	1.294	1.340	−6.39	−9.12	1.43
20	1.097	1.110	−7.53	−10.18	1.35
20	1.097	1.040	−7.53	−9.71	1.29
20	1.097	1.090	−7.53	−9.61	1.28
25	0.865	0.886	−8.97	−10.78	1.20
25	0.865	0.941	−8.97	−10.45	1.16
30	0.661	0.661	−10.40	−11.67	1.12
30	0.661	0.661	−10.40	−11.50	1.11
30	0.661	0.641	−10.40	−11.50	1.11
35	0.490	0.447	−11.83	−12.85	1.09
35	0.490	0.488	−11.83	−12.53	1.06
35	0.490	0.501	−11.83	−12.70	1.07
40	0.353	0.357	−13.27	−14.04	1.06
40	0.353	0.365	−13.27	−13.99	1.05

The buffer was 0.20 M potassium acetate/0.20 M potassium chloride/0.50 M sucrose. The standard deviation in K_B was ±0.043 × 10^5 M^{-1}, ΔC_p for ΔH_{vH} was −0.287 kcal·K^{-1}·mol^{-1}, and ΔC_p for ΔH_{cal} was −0.198 kcal·K^{-1}·mol^{-1}.

In Table 1 are listed the data for 16 calorimetric titrations in the temperature interval 15–40°C. The calculated binding constant (K_B) values given in column 2 agree with the observed values in column 3 with a standard deviation amounting to ≈2% of the value of K_B at 25°C. The calculated values of ΔH_{vH} in column 4 differ markedly from the values observed for ΔH_{cal} in column 5, as illustrated by the ratio of ΔH_{cal} to ΔH_{vH} listed in column 6. The discrepancy between these two enthalpies is highlighted by the fact that ΔC_p as evaluated from the equilibrium constants is −0.287 kcal·K^{-1}·mol^{-1}, which is 45% larger in magnitude than that derived from the observed enthalpies—namely, −0.198 kcal·K^{-1}·mol^{-1}.

To check on the possibility that a significant contribution to ΔH_{cal} might arise from an exchange of protons between the protein and the buffer, the two reactants were mixed in the absence of buffer at pH 5.5 at ≈25°C. The results indicated that the reaction leads to the liberation of 0.025 ± 0.05 mol of H$^+$ per mol of protein, corresponding in acetate buffer to ≈0.05 kcal·mol^{-1} due to buffer protonation and probably a similar, perhaps compensating, contribution due to deprotonation of the protein.

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Table 2. Results from the calorimetric titration of RNase A with 2′-CMP in various buffers at pH 5.5 in the temperature range 10–40°C

Buffer	ΔC_p, kcal·K^{-1}·mol^{-1}		ΔH_{vH},* kcal·mol^{-1}	ΔH_{cal},* kcal·mol^{-1}
	For ΔH_{vH}	For ΔH_{cal}		
a	−0.347	−0.211	−9.63	−12.63
b	−0.265	−0.200	−13.54	−17.65
b′	−0.191	—	−27.70	—
c	−0.287	−0.198	−8.97	−10.62
d	0.0023	−0.258	−7.80	−8.45

Buffers: a, 0.20 M potassium acetate/0.20 M potassium chloride; b, 0.05 M potassium acetate; b′, using UV spectrophotometry instead of titration calorimetry; c, 0.20 M potassium acetate/0.20 M potassium chloride/0.5 M sucrose; d, 0.20 M potassium acetate/0.20 M potassium chloride/1.0 M guanidinium chloride.
*At 25°C.

A check on our procedure for the nonlinear least-squares analysis of temperature–equilibrium constant data is afforded by the accurate ionization constants for diisopropylcyanoacetic acid at 5–45°C reported by Ives and Marsden (2). These authors used a much more elaborate calculational procedure, including ΔC_p with terms up to temperature raised to the third power. Our values for K_{ioniz} differed from theirs by less than 0.1%, and our calculated enthalpies differed from theirs by less than 0.35%.

Table 3. Calorimetric titrations of the S protein of RNase S with truncated S peptides with various substitutions at Met-13

t, °C	$K_B \times 10^5$, M^{-1}		ΔH_{vH}, kcal·mol^{-1}	ΔH_{cal}, kcal·mol^{-1}	$\Delta H_{cal}/\Delta H_{vH}$
	Calculated	Observed			
			Met-13 → Ala		
5.00	1.41	1.54	−16.8	−16.6	0.99
10.00	0.79	0.61	−19.6	−21.3	1.09
14.80	0.42	0.51	−22.3	−23.8	1.07
19.80	0.21	0.21	−25.1	−31.5	1.25
25.00	0.094	0.091	−28.0	−35.3	1.26
			Met-13 → Phe		
5.30	18.0	19.0	−4.4	−18.8	4.27
10.30	13.3	12.4	−15.2	−23.6	1.55
14.90	7.5	7.0	−25.2	−25.6	1.02
20.40	2.7	3.2	−37.2	−30.2	0.81
25.00	0.89	0.82	−47.1	−37.6	0.80
			*Met-13 → α-amino-*N*-butyric acid*		
5.60	102.1	95.2	−13.6	−17.3	1.27
10.00	66.4	78.9	−17.1	−20.3	1.19
14.70	38.2	33.9	−21.0	−23.6	1.12
19.90	18.7	18.4	−25.2	−25.6	1.02
24.90	8.5	8.7	−29.3	−31.8	1.09
			Met-13 → norleucine		
10.0	246.1	250.0	−23.6	−21.6	0.92
10.0	246.1	240.0	−23.6	−21.7	0.92
15.0	117.7	110.0	−24.3	−25.1	1.03
15.0	117.7	130.0	−24.3	−24.9	1.02
20.0	56.5	56.0	−25.0	−27.3	1.09
20.0	56.5	55.0	−25.0	−27.3	1.09
25.0	27.3	29.0	−25.7	−33.5	1.30
25.0	27.3	29.0	−25.7	−35.4	1.38

The standard deviation in K_B was $\pm 0.11 \times 10^5$ M^{-1} for the Met-13 → Ala substitution, $\pm 0.65 \times 10^5$ M^{-1} for the Met-13 → Phe substitution, $\pm 6.7 \times 10^5$ M^{-1} for the Met-13 → α-amino-*N*-butyric acid substitution, and $\pm 5.8 \times 10^5$ M^{-1} for the Met-13 → norleucine substitution. For the Met-13 → Ala substitution ΔC_p was −0.56 kcal·K^{-1}·mol^{-1} for ΔH_{vH} and −0.96 kcal·K^{-1}·mol^{-1} for ΔH_{cal}. For the Met-13 → Phe substitution, ΔC_p was −2.17 kcal·K^{-1}·mol^{-1} for ΔH_{vH} and −0.89 kcal·K^{-1}·mol^{-1} for ΔH_{cal}. For the Met-13 → α-amino-*N*-butyric acid substitution, ΔC_p was −0.82 kcal·K^{-1}·mol^{-1} for ΔH_{vH} and −0.71 kcal·K^{-1}·mol^{-1} for ΔH_{cal}. For the Met-13 → norleucine substitution, ΔC_p was −0.14 kcal·K^{-1}·mol^{-1} for ΔH_{vH} and −0.81 kcal·K^{-1}·mol^{-1} for ΔH_{cal}.

Table 4. Calorimetric titrations of α- and β-cyclodextrins with cyclohexanol

t, °C	K_B, M^{-1}		ΔH_{vH}, kcal·mol^{-1}	ΔH_{cal}, kcal·mol^{-1}	$\Delta H_{cal}/\Delta H_{vH}$
	Calculated	Observed			
			α-Cyclodextrin		
14.9	78.7	78.0	−3.72	−2.39	0.064
22.7	66.0	72.0	−3.93	−2.68	0.68
24.8	63.0	62	−3.98	−3.06	0.77
25.0	62.7	58	−3.99	−3.18	0.80
29.9	56.1	57	−4.12	−3.30	0.80
35.1	49.9	50	−4.26	−3.68	0.86
			β-Cyclodextrin		
20.0	763	771	−2.16	−1.17	0.54
25.0	714	718	−2.44	−1.60	0.66
25.0	714	704	−2.44	−1.58	0.65
25.0	714	732	−2.44	−1.55	0.64
25.0	714	688	−2.44	−1.60	0.66
25.0	714	704	−2.44	−1.58	0.65
30.0	664	687	−2.73	−1.96	0.72
35.0	615	608	−3.01	−2.37	0.79

For α-cyclodextrin, the standard deviation in K_B was ±3.2, ΔC_p for ΔH_{vH} was −0.027 kcal·K^{-1}·mol^{-1}, and ΔC_p for ΔH_{cal} was −0.065 kcal·K^{-1}·mol^{-1}. For β-cyclodextrin, the standard deviation in K_B was ±15, ΔC_p for ΔH_{vH} was −0.057 kcal·K^{-1}·mol^{-1}, and ΔC_p for ΔH_{cal} was −0.079 kcal·K^{-1}·mol^{-1}.

Results obtained in the calorimetric titration of RNase with 2′-CMP in various buffers are summarized in Table 2. Comparison of the data for buffers a and b shows that reduction of the ionic strength and/or of the concentration of phosphate has a remarkable effect, particularly on the enthalpies at 25°C. The results in buffer c indicate that the addition of 0.5 M sucrose has very small effects on the values for ΔC_p but nontrivial effects on the enthalpies. In the presence of 1.0 M guanidinium chloride (buffer d), the van't Hoff enthalpy is constant over the temperature range studied.

We have also evaluated the binding constants, K_B, by spectrophotometry in the UV range in 0.05 M potassium acetate buffer at pH 5.5. Spectrophotometry is admittedly a relatively poor procedure to employ in this case because of the similarity in the UV spectra of 2′-CMP and RNase A. Nonlinear least-squares analysis of the data obtained at 262 nm (Table 2, buffer b′) gave values for ΔH_{vH} that were ≈10 kcal·mol^{-1} more negative than ΔH_{cal} (Table 2, buffer b), and ΔC_p for ΔH_{vH} was −0.191 kcal·K^{-1}·mol^{-1}.

Similar discrepancies have been observed with other systems. For example, Table 3 shows results based on four of the calorimetric studies by Varadarajan *et al.* (3) on the binding of truncated S peptides with various replacements at Met-13 to the S protein of RNase S. As listed in column 6 of Table 3, wide differences between the two types of enthalpies are found for these reactions, with no obvious regularity in the differences.

Analysis of data for the interaction of cyclohexanol with α- and β-cyclodextrins (4) indicates that the discrepancies shown above are not limited to processes involving proteins. The results obtained in these cases are summarized in Table 4 in the same format as employed in Table 1.

The extent of the discrepancies found in the systems studied here may be summarized by noting that the ratio of ΔH_{cal} to ΔH_{vH} at 25°C varied in the range 0.54 to 4.3, and the ratio ΔC_p (cal)/ΔC_p(vH) varied in the range 0.41 to 5.8, if we exclude the value −110 found for 2-CMP binding to RNase A in the presence of 1.0 M guanidinium chloride.

We have analyzed the calorimetric titration data for several other systems, including the binding of various mononucleotides to ribonuclease T_1 (5), the binding of arabinose and

galactose to the arabinose binding protein of *Escherichia coli* (6), and the pairwise interactions of three synthetic oligonucleotides that form a DNA three-way junction (7), and in no case have we found agreement between van't Hoff and calorimetric enthalpies.

The discrepancies noted here are not easily explained in any quantitative way. If we can assume that the concentrations of the substrate (S), the ligand (L), and the product of the binding (i.e., the components of the complex) are low enough so that deviations of these components from ideality can be ignored, then the discrepancies must all be due to changes with temperature in the equilibria controlling the interactions of buffer or solution components, including water, with one or more of the components of the complex. If we denote the equilibrium constant for the main reaction as

$$K_{app} = \frac{[SL]}{[S][L]} \quad [2]$$

and lump all the other unspecified processes into a single constant K', then we may write the true equilibrium constant as

$$K_T = K_{app}K'. \quad [3]$$

The van't Hoff equation is

$$\left(\frac{\partial \ln K_T}{\partial (1/T)}\right)_P = -\frac{\Delta H_{cal}}{T}, \quad [4]$$

where ΔH_{cal} is the actually observed enthalpy change, including all contributions from any processes involving buffer or solution components. Although the analysis given here makes it clear that the true enthalpy change in the actually observed process is indeed ΔH_{cal}, it must be realized that this quantity applies to a complex process including steps that are not indicated by the simple equilibrium $S + L \rightleftarrows SL$ and that are not readily identified. The variation of ΔH_{cal} with experimental conditions as illustrated, for example, in Table 2 further emphasizes the possible complexity of the processes that we actually observe experimentally.

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