

# Van't Hoff analysis of $K^\circ(T)$ : How good...or bad?

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## Abstract

Binding constant data  $K^\circ(T)$  are commonly subjected to van't Hoff analysis to extract estimates of  $\Delta H^\circ$ ,  $\Delta S^\circ$ , and  $\Delta C_p^\circ$  for the process in question. When such analyses employ unweighted least-squares fitting of  $\ln K^\circ$  to an appropriate function of the temperature  $T$ , they are tacitly assuming constant relative error in  $K^\circ$ . When this assumption is correct, the statistical errors in  $\Delta G^\circ$ ,  $\Delta H^\circ$ ,  $\Delta S^\circ$ ,  $\Delta C_p^\circ$ , and the  $T$ -derivative of  $\Delta C_p^\circ$  (if determined) are all independent of the actual values of  $K^\circ$  and can be computed from knowledge of just the  $T$  values at which  $K^\circ$  is known and the percent error in  $K^\circ$ . All of these statistical errors except that for the highest-order constant are functions of  $T$ , so they must normally be calculated using a form of the error propagation equation that is not widely known. However, this computation can be bypassed by defining  $\Delta H^\circ$  as a polynomial in  $(T-T_0)$ , the coefficients of which thus become  $\Delta H^\circ$ ,  $\Delta C_p^\circ$ , and  $1/2 \, d\Delta C_p^\circ/dT$  at  $T=T_0$ . The errors in the key quantities can then be computed by just repeating the fit for different  $T_0$ . Procedures for doing this are described for a representative data analysis program. Results of such calculations show that expanding the  $T$  range from 10–40 to 5–45 °C gives significant improvement in the precision of all quantities.  $\Delta G^\circ$  is typically determined with standard error a factor of  $\sim 30$  smaller than that for  $\Delta H^\circ$ . Accordingly, the error in  $T\Delta S^\circ$  is nearly identical to that in  $\Delta H^\circ$ . For 4% error in  $K^\circ$ , the  $T$ -derivative in  $\Delta C_p^\circ$  cannot be determined unless it is  $\sim 10 \text{ cal mol}^{-1} \text{ K}^{-2}$  or greater; and  $\Delta C_p^\circ$  must be  $\sim 50 \text{ cal mol}^{-1} \text{ K}^{-1}$ . Since all errors scale with the data error and inversely with the square root of the number of data points, the present results for 4% error cover any other relative error and number of points, for the same approximate  $T$  structure of the data.

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## 1. Introduction

Binding and complexation processes are of great interest in many areas of research and are studied by a variety of experimental methods [1–7]. Of these, only the calorimetric approaches provide direct estimates of  $\Delta H$  for the binding process, so when other methods are used, the key thermodynamic properties— $\Delta H^\circ$ ,  $\Delta S^\circ$ , and  $\Delta C_p^\circ$ —are commonly obtained from a van't Hoff (vH) analysis of the temperature dependence of the binding constant  $K^\circ$ . In its differential form, the vH relation can be expressed as

$$\left(\frac{\partial \ln K^\circ}{\partial T}\right)_p = \frac{\Delta H^\circ}{RT^2}. \quad (1)$$

With adoption of a suitable form for  $\Delta H^\circ$ , this equation can be integrated to give an expression appropriate for analysis of  $K^\circ(T)$  by least-squares fitting, from which the desired constants can be extracted. For example, the form [7],

$$\Delta H^\circ = a + b(T - T_0) + c(T - T_0)^2, \quad (2)$$

is sufficiently flexible to permit estimation of the temperature dependence of  $\Delta C_p^\circ$ , since at  $T=T_0$ ,  $\Delta H^\circ=a$ ,  $\Delta C_p^\circ=b$ , and  $d\Delta C_p^\circ/dT=2c$ . Integration of Eq. (1) then yields

$$R \ln(K^\circ/K^\circ_0) = A \left(\frac{1}{T_0} - \frac{1}{T}\right) + B \ln\left(\frac{T}{T_0}\right) + c(T - T_0), \quad (3)$$

where

$$A = a - bT_0 + cT_0^2 \quad \text{and} \quad B = b - 2cT_0. \quad (4)$$

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In practice the vH analysis is often done by fitting  $\ln K^\circ$  to some version of the rearranged form of Eq. (3),

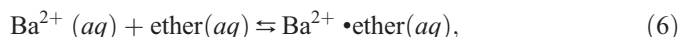
$$R \ln K^\circ = R \ln K_0^\circ + A \left( \frac{1}{T_0} - \frac{1}{T} \right) + B \ln \left( \frac{T}{T_0} \right) + c(T - T_0), \quad (5)$$

in which  $K_0^\circ$  is the fitted value of  $K^\circ$  at  $T_0$ .

Under the usual assumption of error-free independent variable  $T$ , Eq. (5) is actually a linear least-squares fit model, for which the estimation and interpretation of statistical errors for the adjustable parameters is straightforward [8]. Yet surprisingly, many published vH analyses seem to omit these important quantities. Those that do report the parameter errors do so inadequately. That is because unless  $c=0$ , the error in  $\Delta C_P$  is a function of  $T$ ; and unless both  $b$  and  $c=0$ , the error in  $\Delta H^\circ$  is a function of  $T$ . The purpose of the present work is to emphasize two points: (1) In the usual approach of unweighted fitting of  $\ln K^\circ$ , where the tacit assumption is constant relative error in  $K^\circ$ , the statistical errors in the adjustable parameters are independent of the values of  $K$  and depend only on the  $T$  structure of the data and the scale of the data error. (2) Although proper assessment of the  $T$ -dependent errors in  $\Delta H$  and  $\Delta C_P$  normally requires use of a version of the error propagation formula that is not familiar to many workers, this can be bypassed by simply repeating the fit to Eq. (5) for different  $T_0$ . Procedures for accomplishing this are illustrated with a widely used data analysis program, KaleidaGraph (Synergy Software) [9].

Among the results of this study are the realization that quite high precision in  $K^\circ$  ( $\sim 1\%$ ) may be required to estimate the  $T$ -dependence of  $\Delta C_P$  with statistical significance; and even  $\Delta C_P$  itself is often not well-determined for typical precision in  $K^\circ$ . On the other hand, neglect of these constants, even when statistically insignificant, can lead to appreciable bias in the estimates of  $\Delta H^\circ$  and  $\Delta S^\circ$ . These points are illustrated with reference to two representative sets

of  $K$  data—one for a typical enthalpy-driven binding process [7],



and one more characteristic of competitive enthalpy/entropy effects, as occur in many biochemical processes [6]. These are illustrated in Fig. 1 and discussed further below.

## 2. Methods

### 2.1. Statistical error propagation

The textbook expression,

$$\sigma_f^2 = \sum \left( \frac{\partial f}{\partial \beta_j} \right)^2 \sigma_{\beta_j}^2, \quad (7)$$

is normally used to compute the propagated error in some function  $f$  of the independent variables  $\beta$ , where the sum runs over all uncertain variables  $\beta_j$ . However, Eq. (7) assumes that these variables are uncorrelated. This assumption seldom holds for a set of parameters  $\beta$  returned by an LS fit, and one must use the more general expression,

$$\sigma_f^2 = \mathbf{g}^T \mathbf{V} \mathbf{g}, \quad (8)$$

in which the  $j$ th element of the vector  $\mathbf{g}$  is  $\partial f / \partial \beta_j$ , and  $\mathbf{V}$  is the variance–covariance matrix (more below). This expression is rigorously correct for functions  $f$  that are linear in variables  $\beta_j$  that are themselves normal variates [10]. For nonlinear functions of normal and nonnormal variates, its validity is only approximate, but it usually follows the same 10% rule of thumb that applies to parameters estimated by nonlinear LS [11]: If the  $\mathbf{V}$ -based standard error is less than 1/10 the magnitude of the parameter, then the Gaussian interpretation of this error should suffice within 10% to define the confidence limits. Often the computation of  $\sigma_f$  can be facilitated by simply redefining the fit function so that  $f$  is one of the adjustable parameters of the fit [10].

As a simple example to illustrate the differences between Eqs. (7) and (8), consider the straight line model,  $y = a + bx$ . The  $\mathbf{V}$  matrix is  $2 \times 2$ , with diagonal elements  $V_{11} = \sigma_a^2$  and  $V_{22} = \sigma_b^2$ , and off-diagonal  $V_{12} = V_{21} = \sigma_{ab}^2$ . If we take as the function  $f(x)$   $y$  itself, we find from Eq. (8)

$$\sigma_f^2 = \sigma_a^2 + \sigma_b^2 x^2 + 2\sigma_{ab}^2 x, \quad (9)$$

while Eq. (7) misses the last term in (9), which contains the correlation between  $a$  and  $b$ .

As already noted, if there is no correlation, Eq. (7) suffices. An important example is the transformation of an uncertain variable to another form for fitting. Thus, if  $z = \ln y$ , we find that  $\sigma_z = \sigma_y / y$ . Now if  $y$  has constant *relative* error, i.e.,  $\sigma_y = r y$ , the error in  $z$  becomes constant,  $\sigma_z = r$ . The method of least squares yields minimum variance estimates of the parameters only if the data are weighted as their inverse

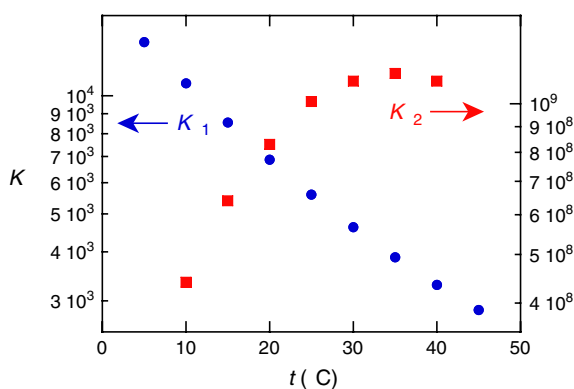


Fig. 1. Logarithmic display of binding constants for a typical enthalpy-driven process ( $K_1$ , round, from Ref. [7], scale to left) and a process with competitive enthalpy and entropy effects ( $K_2$ , square, from Fig. 1 in Ref. [6]). For these models, the values of the key parameters at 25 °C are as follows:  $K_1: K_0^\circ = 5600$ ,  $\Delta H^\circ = -7.0$  kcal/mol;  $\Delta C_P^\circ = 50$  cal mol $^{-1}$  K $^{-1}$ , and  $c = 1$  cal mol $^{-1}$  K $^{-2}$ ;  $K_2: K_0^\circ = 1 \times 10^9$ ,  $\Delta H^\circ = 5.0$  kcal/mol;  $\Delta C_P^\circ = -530$  cal mol $^{-1}$  K $^{-1}$ , and  $c = 2$  cal mol $^{-1}$  K $^{-2}$ .

variances. Thus in the latter case, the properly weighted fit of  $z$  as dependent variable involves constant weights, making it de facto an unweighted fit. This is the reason for the earlier statement that unweighted fits of  $\ln K$  tacitly assume constant relative error in  $K$ .

There has been at least one treatment of the van't Hoff analysis with attention paid to error propagation in the estimation of  $\Delta H^\circ$ , but apparently with neglect of the correlation terms [12]. The significance of such neglect will be addressed below, but it should be noted that sometimes the correlation can be rigorously reduced to zero, making Eq. (7) correct. For example, if the fit to a straight line be recast as  $y = a + b(x - x_0)$ , then in an unweighted fit  $\sigma_{ab}^2$  vanishes when  $x_0$  is taken as the mean  $x$  value. For any value of  $x_0$ ,  $y = a$  at  $x = x_0$ ; so  $\sigma_f^2 = \sigma_a^2$  at this point. This is an example of bypassing Eq. (8) to properly compute the error in a desired quantity (here the fit function itself) through a redefinition of the fit parameters. In the present case of interest, the same can be accomplished by simply repeating the fit to the model of Eq. (5) for different  $T_0$  over the range of interest (which need not be restricted to the range of the actual  $T$  values).

## 2.2. Variance–covariance matrices— $V_{\text{prior}}$ and $V_{\text{post}}$

As already indicated, the variance–covariance matrix  $\mathbf{V}$  is key to the assessment of statistical errors in the parameters and their functions, with the diagonal elements of  $\mathbf{V}$  being the parameter variances and the off-diagonal their covariances. Without going into detail about the mechanics of least-squares computations, which are fully described in many sources [8,11,13,14], it is nonetheless important to distinguish two versions of the  $\mathbf{V}$  matrix, which I have referred to as *a priori* ( $V_{\text{prior}}$ ) and *a posteriori* ( $V_{\text{post}}$ ). Both are proportional to the inverse matrix  $\mathbf{A}^{-1}$ , where

$$\mathbf{A} = \mathbf{X}^T \mathbf{W} \mathbf{X}. \quad (10)$$

This is given here in this form to call attention to the role of the weight matrix  $\mathbf{W}$ . If there are  $n$  points in the data set,  $\mathbf{W}$  is  $n \times n$ ; and in the vast majority of situations, where the data are uncorrelated,  $\mathbf{W}$  is diagonal, with elements  $W_{ii} = w_i = 1/\sigma_i^2$ ,  $\sigma_i$  being the standard deviation in  $y_i$ . In the simple case where all  $\sigma_i$  are the same, ( $\sigma_i = \sigma$ ),  $\sigma^{-2}$  factors out of  $\mathbf{W}$ , and  $\mathbf{A}^{-1}$  becomes proportional to  $\sigma^2$ . The same relationship holds for nonconstant  $\sigma_i$ : Namely, if all  $\sigma_i$  are scaled by a factor  $f$ , then  $\mathbf{A}^{-1}$  is scaled by a factor  $f^2$ . This is important, because

$$\mathbf{V}_{\text{prior}} = \mathbf{A}^{-1}, \quad (11)$$

from which we see that the parameter standard errors scale with the data error (and likewise for all propagated errors).

$V_{\text{prior}}$  is appropriate in those cases where the data error is known absolutely in advance, and it is exact for linear fit models in that case. An obvious example is Monte Carlo computations on a fit model, where the computationalist sets the data error. However, strong arguments can be made for

the use of  $V_{\text{prior}}$  in many experimental situations where each run involves a small number of data points, but where archival data for the same equipment and procedures arguably provide a better determination of the data error than does the single experiment in question [15]. Nonetheless, the firmly ensconced default in most of physical science is  $V_{\text{post}}$ , defined as

$$\mathbf{V}_{\text{post}} = \frac{S}{v} \mathbf{A}^{-1}, \quad (12)$$

where  $v$  is the number of degrees of freedom, equal to the number of data points  $n$  minus the number of adjustable parameters  $p$ ; and  $S$  is the sum of weighted squared residuals,  $= \sum w_i \delta_i^2$ , with the residual  $\delta_i$  being the difference between measured and fitted values of the dependent variable ( $\ln K_i$  in the fit to Eq. (5)).

In the common case of an unweighted fit, where all  $w_i = 1$ , the prefactor in Eq. (12) is an estimate of the variance in the dependent variable,  $s_y^2 = S/v$ . Note, however, that if the data are not truly of constant uncertainty, the unweighted fit will not yield minimum-variance parameter estimates. In other words, the error structure of the data needs to be known to within a scale factor for the fit to yield minimum-variance estimates. Then in the more general case of nonconstant weights, the quantity  $S/v$  becomes an estimate of the variance for data of unit weight.

When the data errors are known absolutely (making  $V_{\text{prior}}$  appropriate),  $S/v$  is a sampled estimate of the reduced  $\chi^2$ , which has average value 1 and variance  $2/v$ . All of the elements of  $V_{\text{post}}$  behave as scaled  $\chi^2$  variates, which means they are subject to the same statistical properties. Thus a parameter error estimated from  $V_{\text{post}}$  has relative uncertainty  $(2v)^{-1/2}$ . For example, a van't Hoff analysis via Eq. (5) with 7 data points has  $v = 3$ ; hence the  $V_{\text{post}}$ -based parameter errors are uncertain by 40% (and far from Gaussian in their distribution for such small  $v$ ).

In results given below, I will use  $V_{\text{prior}}$  to discuss results as a function of the presumed data error. Although it is easy to obtain the key results without ever actually computing  $V_{\text{prior}}$ , it is still necessary to understand the distinction, in order to make proper use of available data analysis programs. For example, when weighted fitting is invoked with the KaleidaGraph program, the user must provide a column of  $\sigma_i$  values; and the program treats these as absolute, so that the resulting parameter standard errors are  $V_{\text{prior}}$ -based. If the weights are intended to be valid in only a relative sense (as from a linear-to-log data transformation), the user must scale the reported errors by  $(S/v)^{1/2}$ . [ $S$  is called “chi-square” in the program but is not truly  $\chi^2$  unless the data weights are valid absolutely.] In the Origin program (OriginLab) widely used to analyze ITC data, the default is again  $V_{\text{prior}}$ ; but the user may check a box “Scale errors with  $\sqrt{\text{reduced } \chi^2}$ ” to convert to *a posteriori*.

It is worth noting that  $V_{\text{prior}}$  can be used with confidence to obtain statistical errors for exactly fitting data, where  $S = 0$ . Of course  $V_{\text{post}}$  gives meaningless results in this situation.

### 2.3. Least-squares fitting procedures

The fitting is easily done using programming languages or with any of a number of available data analysis and presentation programs, like the KaleidaGraph and Origin programs already mentioned, and others like SigmaPlot and IgorPro. These have options that permit users to define their own fit models through entry in a statement box or Macro Library. Eq. (5) is simple enough to be entered directly, but there are advantages to using multistep definitions in a Macro Library, as available in the KaleidaGraph (KG) program [9]. For example, to fit  $\ln K^\circ$  using KG, one can enter the following in the library:

```
T0 = 283.15;
RG = 1.9872;

dh = a;
dcp = b;
ddcp = c;

AA = (dh - dcp*T0 + ddcp*T02);
BB = (dcp - 2*ddcp*T0);

VLK(x) = (AA*(1/T0 - 1/x) + BB*ln(x/T0) + ddcp*(x - T0))/RG;
```

(13)

and then enter in the “define fit” box,

```
ln(d) + VLK(x); a = 1e4; b = 100;
c = 1; d = 5e3
```

(14)

where the entries after the first semicolon are initial estimates, required at least the first time the fit is run (but the precise values of which are not very important in this case). In the form of Eq. (14), the fit is actually nonlinear, because of the way in which  $K^\circ_0$  is defined (as  $d$ ); however the other adjustable parameters ( $a$ ,  $b$ ,  $c$ ;  $a$ – $d$  are assigned as adjustable parameters by default in the KG program) are truly linear and thus behave as predicted for linear parameters under the usual assumptions behind LS fitting [8]. The advantage of defining the fitted quantity as  $\ln(d)$  instead of just  $d$  is that with the former, the fit returns directly the fitted value of  $K^\circ_0$  and its standard error. The reason for specifically defining  $dh$ ,  $dcp$ , and  $ddcp$  in Eq. (13) in terms of the default adjustable parameters ( $a$ ,  $b$ , and  $c$ ) is that in this form, these can easily be set to a constant value (like 0) if desired.

If the fit is run as defined in Eqs. (13) and (14), the output will be the values of the parameters and their errors at 10 °C ( $T_0 = 283.15$ ), and the units will be in cal ( $RG = 1.9872$ ). Rerunning the fit for different defined  $T_0$  will yield statistically identical results, but different values for the parameters and their errors (except for  $c$  and its error, which are independent of such changes; or  $b$  and its error, if  $c$  is set to 0). In this way, one bypasses the computation of  $V$  and the use of Eq. (8) while

still producing fully correct parameter and error estimates at each  $T_0$ . One can compute  $\Delta G^\circ$  at each  $T_0$  using

$$\Delta G^\circ = -RT_0 \ln K^\circ, \quad (15)$$

and then its error from Eq. (7),

$$\sigma_{\Delta G^\circ} = RT_0 \sigma_{K^\circ} / K^\circ \quad (16)$$

Note that when  $\sigma_{\Delta G^\circ}$  is computed this way from the fitted values of  $K^\circ$  and its standard error, it will be smaller than when evaluated directly from the original  $K^\circ$  values and their errors. This is the result of the “averaging” effect of the fit, analogous to the distinction between the standard deviation and the standard deviation in the mean (standard error) when a single quantity is averaged  $n$  times. The resulting  $\sigma_{\Delta G^\circ}$  will also be a function of  $T_0$ , achieving its minimal value near the middle of the  $T$  range.

As an alternative to Eqs. (15) and (16), identical results can be obtained by redefining the fit function in Eq. (14) as

$$-d/RG/T_0 + \text{VLK}(x); \quad (17)$$

in which the adjustable parameter  $d$  is now  $\Delta G^\circ$  itself. The other key parameter of interest from a van't Hoff analysis is  $\Delta S^\circ$  (or  $T\Delta S^\circ$ ), which can be obtained directly, with its correct statistical error, through yet another definition of the fit function:

$$(d - dh)/RG/T_0 + \text{VLK}(x); \quad (18)$$

Now  $d$  is  $T\Delta S^\circ$ , and the fit returns correct values for it and its error at each  $T_0$ . In a further effort saver, all three variations—Eqs. (14), (17), and (18)—can be defined and run simultaneously for each new value of  $T_0$ .

### 3. Results and discussion

I have chosen to illustrate results of such computations for 4% error in the binding constant. This is roughly a geometric mean of values encountered in practice, which can be as much as an order of magnitude smaller in best cases, or an order of magnitude larger in worst. As has already been noted, since the fit is linear (for all except  $d$  in Eq. (14)), the results for the

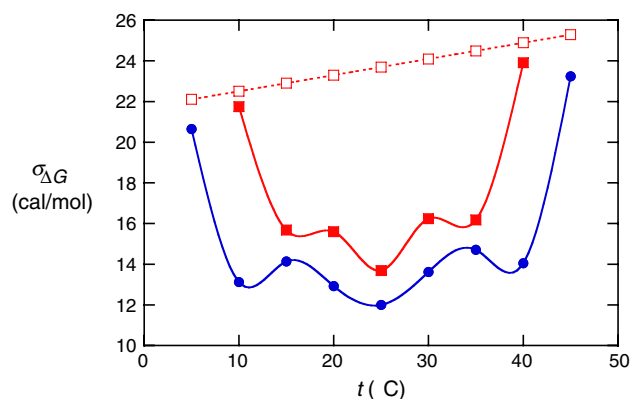


Fig. 2. Statistical error in  $\Delta G^\circ$  for 4% error in  $K^\circ$  and 7 evenly spaced  $T$  values between 10 and 40 °C (square) or 9  $T$  values between 5 and 45 °C (round). Also shown (open squares and dashed line) are the “raw” errors from the individual data values.



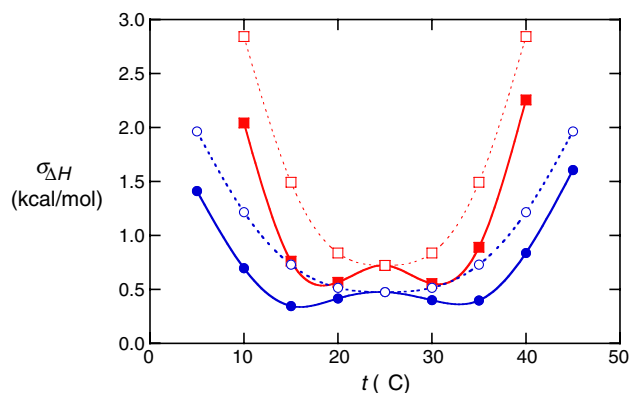


Fig. 3. Statistical error in  $\Delta H^\circ$  from van't Hoff analysis, identified as in Fig. 2. In addition are shown the "naive" propagated errors (open points and dashed curves), obtained using Eq. (7) with the parameter errors obtained at 25 °C.

statistical errors in  $\Delta H^\circ$ ,  $\Delta C_p^\circ$ , and  $\Delta G^\circ$  are independent of the  $K^\circ$  values. They do depend on the choice of 4% error and on the chosen  $T$ -structure of the data. However, if the latter is fixed, results for different percent error are obtained simply by scaling, as a consequence of the previously noted fact that the parameter errors scale with the data error.

Results are shown in Figs. 2–4 for  $T$  values at 5 °C intervals between 10 and 40 °C, and for a slightly expanded range having one extra point at each end. The expansion achieves significantly improved precision in  $\Delta H^\circ$  and  $\Delta C_p^\circ$  over the full range. This result is not just a consequence of the increase in  $\nu$  from 3 to 5, because identical results are obtained for four values at each  $T$ , having 8% error each (giving 4% average error, from the  $1/\sqrt{n}$  dependence of the standard error), for which the  $\nu$  values of relevance are 24 and 32. The statistical error in  $c$  is 11.5 cal mol<sup>-1</sup> K<sup>-2</sup> for the 7-point data set, dropping to 4.5 for the 9-point set. Thus  $c$  is statistically undefined for both models used here. Dropping it from the fit model yields improved precision in  $\Delta C_p^\circ$  ( $\sigma=62$  and 32 cal mol<sup>-1</sup> K<sup>-1</sup>), which now becomes constant in the fit. There is also improved precision in  $\Delta H^\circ$ , as illustrated in Fig. 5, which includes the bias that results from setting  $c=0$ .

From the foregoing, the  $T$  derivative of  $\Delta C_p^\circ$  must be at least of order 10 cal mol<sup>-1</sup> K<sup>-2</sup> for it to be estimated from 9  $K^\circ$  values at 5–45 °C, having 4% error. In the likely event that it is

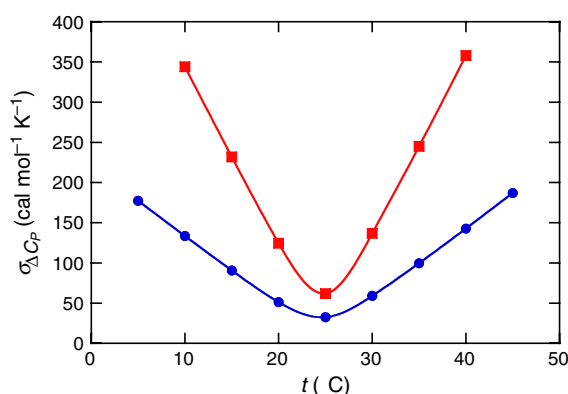


Fig. 4. Statistical error in  $\Delta C_p^\circ$  from van't Hoff analysis, identified as in Figs. 2 and 3.

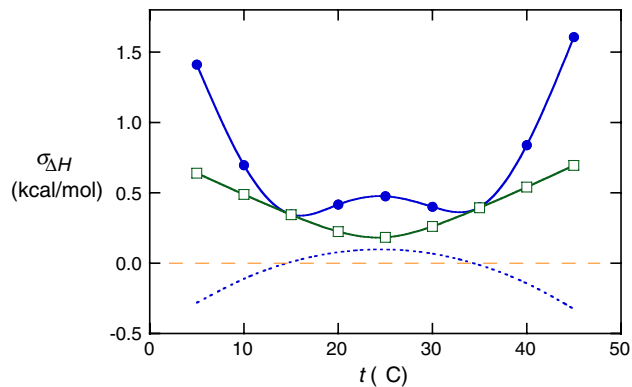


Fig. 5. Effects on the statistical error in  $\Delta H^\circ$  of setting  $c=0$  for the 9-point data set. Solid points are as shown in Fig. 3. Open points represent the values obtained from the 3-parameter fit. The dashed curve shows the bias (fit—true) in  $\Delta H^\circ$  from the latter.

not this large, so that it is omitted from the fit model,  $\Delta C_p^\circ$  itself must be  $\sim 50$  cal mol<sup>-1</sup> K<sup>-1</sup> to be obtained with statistical significance. Fig. 6 shows the significance of these points for the two cases examined here. Both  $\Delta C_p^\circ$  functions can clearly accommodate a constant value within their error bars for the original fit; and when this is done, the precision in  $\Delta C_p^\circ$  becomes constant at very near its previous minimum value.  $\Delta C_{p,2}^\circ$  is everywhere statistically significant in both models, but  $\Delta C_{p,1}^\circ$  is only marginally defined near its midpoint in the  $T$ -dependent model, and then barely significant everywhere with  $\Delta C_p$  taken as constant.

The dashed curves in Fig. 3 show that the error in  $\Delta H^\circ$  is overestimated when it is evaluated using the naive error propagation formula of Eq. (7), the maximum effect being a factor of  $\sim 2$  near 15 and 35 °C. However, for many applications this magnitude of overassessment is not critical. The relatively small excess is a consequence of the use of parameter errors obtained near the middle of the  $T$  range, where the interparameter correlation is not large. If the calculation with Eq. (7) were done with errors estimated near the ends of the  $T$  range, the naive error propagation formula would be worthless. By contrast, Eq. (8) yields correct results for any  $T_0$ . Of course, simply stating the

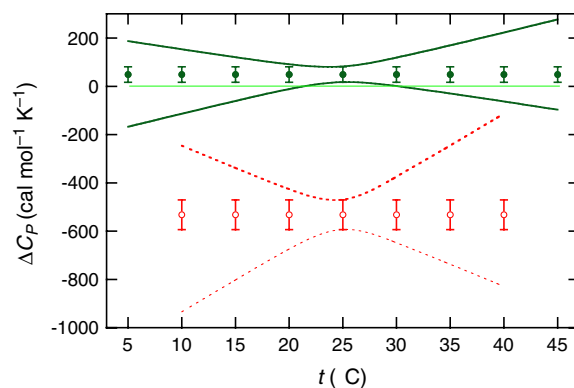


Fig. 6.  $\Delta C_p^\circ$  from van't Hoff analyses of  $K^\circ_1$  (upper) and  $K^\circ_2$ . The curves delimit the  $\pm\sigma$  error bands from fits with  $T$ -dependence in  $\Delta C_p^\circ$  in the model. The points with error bars represent the results with assumption of constant  $\Delta C_p^\circ$ . Note the larger range and number of points for  $K^\circ_1$  than for  $K^\circ_2$ .

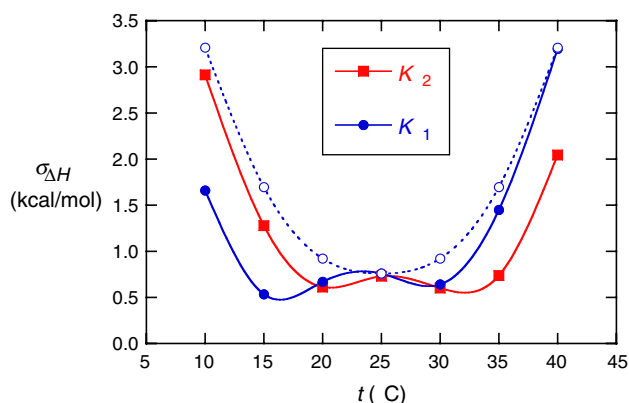


Fig. 7. Statistical error in  $\Delta H^\circ$  for constant error in  $K^\circ$ . For reference to Fig. 3, the  $\sigma_{K^\circ}$  values were taken as the averages of the 4% errors for each  $K^\circ$ , giving roughly the same propagated errors at 25 °C. For this reason, the naive errors (dashed) are nearly identical for  $K_1$  and  $K_2$ .

errors at 25 °C without noting their expansion at the limits of the  $T$  range is grossly misleading.

It is noteworthy that the statistical error in  $\Delta G^\circ$  is a factor of  $\sim 30$  smaller than that in  $\Delta H^\circ$ . Because of this, it is not necessary to display the error in  $T\Delta S^\circ$ , because it is almost identical to that in  $\Delta H^\circ$ . For both of these quantities the statistical error increases significantly (factor  $\sim 4$ ) on moving from the mid-range of  $T$  to the limits of the data range.

If we consider the other common extreme of data error—constant absolute error in  $K^\circ$ —the results depend on the actual  $K$  values. In simplest terms, this is because the weights in the log fit are no longer constant, so different ranges of  $T$  are favored for different dependence of  $K$  on  $T$ . This point is illustrated for  $\Delta H^\circ$  in Fig. 7. Note that if the uncertainties are truly constant, independent of  $K^\circ$ , the proper unweighted fit is to the exponential form of Eq. (5). With proper weighting, both forms give the correct standard errors for both constant absolute and constant relative data error.

Since the results shown for constant relative error in  $K^\circ$  in Figs. 2–6 do depend on the specific  $T$  values at which  $K^\circ$  is obtained, we can ask if some pattern other than evenly spaced values might achieve better precision in the vH analysis. Without examining this question exhaustively, I have looked at distributions of  $T$  that were more compressed and more spread in the mid-range. On examining results for 7 points spanning the range 10–40 °C, I found that such changes could improve precisions in some ranges and worsen them in others; and they could help one parameter and hurt another. However, no unevenly spaced distribution of  $T$ s examined to date has given significantly better results than the evenly spaced one. By extension, we can anticipate that adding more points throughout the  $T$  range will, in first approximation, just lower the errors in accord with the  $1/\sqrt{n}$  dependence.

#### 4. Conclusion

Binding constant data  $K^\circ(T)$  are commonly analyzed by fitting  $\ln K^\circ$  to an appropriate function of  $T$ , as defined by the van't Hoff relation. Most often, this is done with unweighted

least squares, which tacitly assumes constant relative error in  $K^\circ$  at all  $T$ . If this assumption is justified, the statistical errors in  $K^\circ$  (fitted),  $\Delta G^\circ$ ,  $\Delta H^\circ$ ,  $\Delta S^\circ$ , and  $\Delta C_p^\circ$  can be computed, for all time, from just the knowledge of the  $T$ -structure of the data set and the relative error in  $K^\circ$ . Since the standard errors also scale with the data error, results for different data error are immediately obtainable from those for any selected error. Here I have illustrated these points by computing the statistical errors as functions of  $T$ , for 4% data error and (1) 7  $K^\circ$  values evenly spaced at temperatures between 10 and 40 °C, and (2) 9 values between 5 and 45 °C. The expanded  $T$  range gives significantly better precision, so by extension, workers should strive for the greatest feasible  $T$  range in recording  $K^\circ(T)$  for the purpose of a van't Hoff analysis.

One can ask how good is the assumption of constant relative error in  $K^\circ$ . Certainly there are cases where it is quite good. For example, in a recent study I have found that the optimal choice of experimental parameters for ITC leads to roughly constant relative error in  $K^\circ$  over much of the range of  $K^\circ$  that is accessible to this technique [16]. Since the  $K^\circ$  values of interest in biochemical processes typically span only a factor of  $\sim 10$  in magnitude over the accessible  $T$  range, mild deviation from constant relative error does not greatly invalidate the results obtained under the constant relative error assumption, as was seen in Fig. 7 for the case of constant error (which is probably the most extreme violation of the relative error assumption). Thus the present results should remain a useful guideline. The computational methods described here readily yield more precise answers in areas of question.

Given that the  $T$ -derivative of  $\Delta C_p^\circ$  will often not be defined statistically (i.e., will differ from zero by less than one  $\sigma$ ), and that sometimes the same will hold for  $\Delta C_p^\circ$  itself, how should these quantities be treated in the fit model? For estimating the better defined quantities ( $\Delta H^\circ$  and  $\Delta S^\circ$ ), leaving the poorly defined parameters in the fit model has the merits of avoiding bias and of yielding conservative (hence “safer”) error estimates. Of course the best solution is to be able to fix these with some measure of confidence from other work, which will then yield significantly improved precision for the remaining parameters. In terms of the algorithm given in Eq. (13), assigning fixed values to  $\Delta C_p^\circ$  and/or its  $T$ -derivative is done by simply altering the right-hand sides of the relevant assignment statements.

All of the present work on the van't Hoff analysis and apparently almost all in the literature employ either Eq. (1) or its modified version, in which  $\ln K^\circ$  is differentiated with respect to  $(1/T)$ . Interestingly, these forms are actually not quite correct for the concentration reference states that are generally employed in biochemical work. The correct form contains a term in the thermal expansivity of the solvent,  $\alpha(T)$  [17],

$$\left(\frac{\partial \ln K^\circ}{\partial T}\right)_p = \frac{\Delta H^\circ}{RT^2} + \alpha(T). \quad (19)$$

In our recent study of the  $\text{Ba}^{2+}$ /crown ether complexation in water [7], which spanned roughly the larger 5–45 °C range examined here, taking the thermal expansivity term into account altered the vH estimates by almost 2% at the  $T$  extremes. However, if the temperature dependence of the density of water

was neglected in both the computation of the  $K$ s and in the vH analysis, there was a cancellation effect, leading to virtually no systematic error in the vH results for  $\Delta H^\circ$ . The other side of the coin is that when workers take care to include solvent density effects in their  $T$ -dependent  $K$ s, they should analyze the data using Eq. (19) instead of Eq. (1).

The just-mentioned problem is one of systematic error, and it serves to remind us that all of the work in this paper up to the foregoing paragraph has been concerned with random statistical error only, with the partial exception of the attention to bias from deleting poorly defined parameters from the fit model. All of the best effort to minimize and properly quantify statistical error can be rendered moot by remaining systematic errors...which is why persistent small discrepancies between direct and vH estimates of  $\Delta H^\circ$  in ITC remain of interest [7].

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