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Designing isothermal titration calorimetry experiments for the study of 1:1 binding: Problems with the "standard protocol"

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ABSTRACT

Literature recommendations for designing isothermal titration calorimetry (ITC) experiments to study 1:1 binding, M + X ⇄ MX, are not consistent and have persisted through time with little quantitative justification. In particular, the "standard protocol" employed by most workers involves 20 to 30 injections of titrant to a final titrant/titrand mole ratio (R_m) of ~ 2—a scheme that can be far from optimal and can needlessly limit applicability of the ITC technique. These deficiencies are discussed here along with other misconceptions. Whether a specific binding process can be studied by ITC is determined less by c (the product of binding constant K and titrand concentration $[M]_0$) than by the total detectable heat q_{tot} and the extent to which M can be converted to MX. As guidelines, with 90% conversion to MX, K can be estimated within 5% over the range 10 to 10^8 M^{-1} when $q_{tot}/\sigma_q \approx 700$, where σ_q is the standard deviation for estimation of q. This ratio drops to ~ 150 when the stoichiometry parameter n is treated as known. A computer application for modeling 1:1 binding yields realistic estimates of parameter standard errors for use in protocol design and feasibility assessment.

In the study of 1:1 binding, M + X ⇄ MX, by isothermal titration calorimetry (ITC),[1] the titrant X is injected sequentially into a cell containing the titrand M [1]. Each injection produces a signal peak, which returns to baseline after a time of order minutes. The heat q for that injection is obtained by first estimating the baseline and then integrating the signal–baseline difference over the duration of the peak. The full sequence typically consists of 20 to 30 such individual experiments, in which fresh titrant is injected into the cell containing the mix of reactants and product from all preceding injections, assumed to have fully equilibrated prior to the current injection. The data are analyzed using nonlinear least squares (LS) to obtain estimates of the reaction enthalpy ΔH° and the equilibrium binding constant K. Usually one additional parameter is fitted—the stoichiometry number n, which corrects for unknown purity of titrand but also absorbs errors in the presumed active volume V_0 of the cell [2]. A display of q_i versus injection number i or versus the reaction stoichiometry ratio R (the concentration ratio of total titrant to total titrand in the cell, $[X]_0/[M]_0$) varies in shape (Fig. 1), depending on the quantity $K[M]_0$, designated c following Wiseman and coworkers [1]. In simplest terms, the shape of this curve determines K, and the scale determines ΔH°.

The user planning such an experiment must choose concentrations for the two reagents and decide on the number m and volume v of injections. Over the years, prevailing wisdom has dictated that c should be in the range 5 to 20, 10 to 100, >40, 50 to 500, 1 to 1000, or even 0.1 to 1000 [1,3–10]. This recommendation is usually made as part of what I call the "standard protocol"—approximately 25 injections to a titrant/titrand stoichiometry ratio R_m of ~ 2 after the last (mth) injection and including a "throwaway" small first injection. Although the majority of users continue to follow this protocol, it has been pointed out that good results have been obtained for c values much smaller than 1 [11], the primary requirement for success being that the titration range be increased over the standard $R_m = 2$ to achieve adequate conversion of titrand to product. Thus, potential ITC users accepting the guidelines of the standard protocol might wrongly forgo use of the method for situations where it can yield results even better than in most of the so-called optimal regions of c.

In a statistical study of the dependence of parameter precision on R_m, m, c, and a new parameter describing the heat content of the reaction, $h = \Delta H^\circ [M]_0$, I quantified the need for increasing R_m with decreasing c and obtained the expression,

$$R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c}, \text{ but at least } 1.1, \quad (1)$$

for setting it [12]. I also found that near-optimal results could be obtained with just 10 injections, and for low-c and low-q situations, even better results with still fewer injections of variable volume

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[1] *Abbreviations used:* ITC, isothermal titration calorimetry; LS, least squares; SE, standard error; MC, Monte Carlo; RSE, relative standard error.

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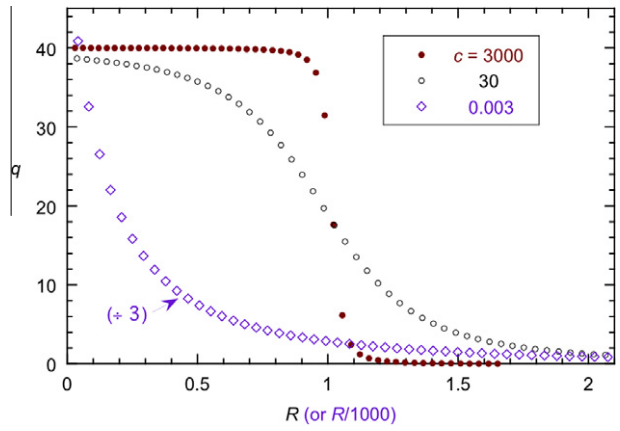

Fig.1. Heat (q) as a function of titration range for 1:1 binding, 50 injections, and selected values of $c = K[M]_0$. The heat scale is arbitrary but set to give the same total reaction heat at completion for the three cases. For $c = 0.003$, the range is compressed by a factor of 1000 and the heat is scaled down for display.

[13] to more evenly distribute the heat for titration curves like that for $c = 0.003$ in Fig. 1. Although successful analysis of low-c data may require that either n or ΔH° be frozen at a known or presumed value, K is decoupled from the other parameters in this regime [14] and can be determined reliably independent of the inherent uncertainty in n and ΔH° (which are very highly correlated at low c [12]).

To illustrate some of these points, I show in Fig. 2 the relative precision in K from LS analysis of synthetic data for typical values of ΔH° and $[M]_0$ and varying K. For the standard protocol ($R_m = 2$), optimal results are obtained near $c = 20$, and K can be estimated within 10% for $c = 1$ to 5500 (or 5% for $c = 3$–400). However, by using Eq. (1) to set the titration range, we obtain better precision in K for all c in the range 0.001 to 400, and the optimal region becomes $c < 10$. Yet this display does not satisfy the real needs of the user, who wants to know how to achieve good results for a given reaction. There the answer in first approximation is to maximize the heat content h by making $[M]_0$ as large as feasible without exceeding reagent solubilities, the instrumental dynamic range, or other high-concentration complications characteristic of the reagents under study. In the example of Fig. 3, precision improves

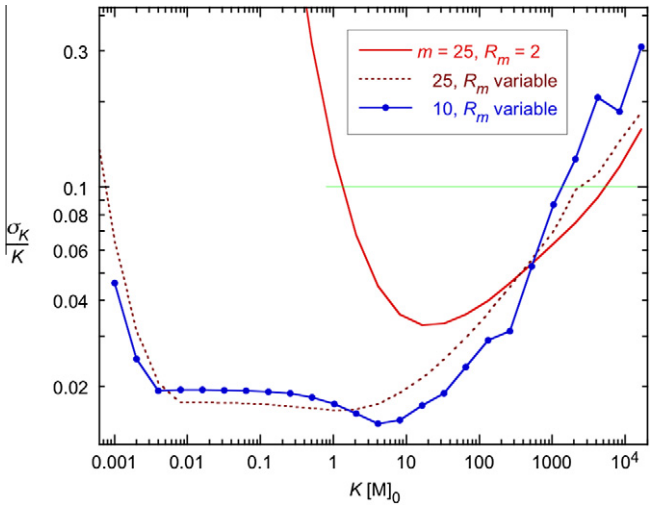

Fig.2. RSE in K as a function of $c = K[M]_0$ for $\Delta H^\circ = 10$ kcal/mol, $[M]_0 = 0.1$ mM, a 5.6:1 cell volume/total titrant volume ratio, and the indicated numbers of injections and titration ranges. All three parameters are fitted. The upturn at small c results from a limit of 1 M placed on the titrant concentration in the syringe.

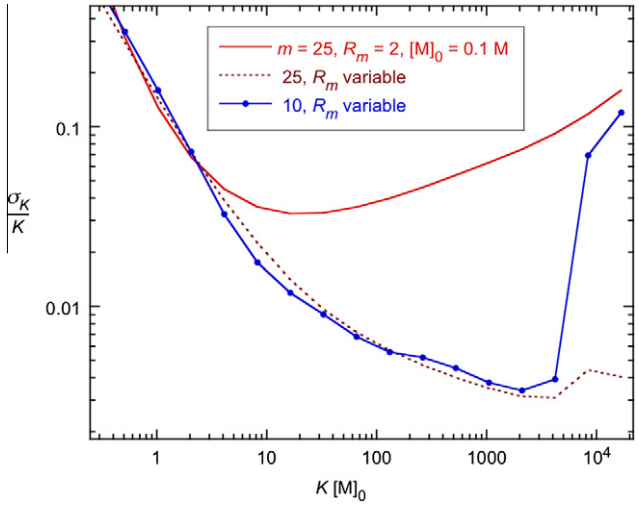

Fig.3. RSE in K versus c for $\Delta H^\circ = 10$ kcal/mol, $K = 10^5$ M^{-1}, and varying $[M]_0$. Other quantities are as in Fig. 2, from which the first curve ($[M]_0$ fixed, K varying) is repeated for reference. Note that h (cal/L) is numerically equal to c/10.

with increasing $[M]_0$ to $c \approx 4000$ before diminishing. However, the corresponding $[M]_0$ is ~40 mM, which is impractically large for many biochemical processes. Realistically, because systematic errors often dominate for statistical precisions better than ~3% [15,16], the user need only make the concentrations large enough to achieve this or any other chosen precision. For example, 4% precision in K is achieved here for $[M]_0 = 40$ μM.

In subsequent sections, I review the computational methods needed to produce curves like those in Figs. 2 and 3. I then discuss why larger R_m and smaller m give better results for low c. I also consider a number of literature assertions that are not supported by quantitative computations, and I reexamine the question of data error and the need for weighted LS in analyzing ITC data. Furthermore, I discuss unresolved questions concerning the active volume and the nature of mixing in perfusion-type instruments. For very-low-c work, I provide prescriptions for variable-volume protocols having more injections than the 4 or 5 given in Ref. [13], to provide extra redundancy with modest loss of precision. Finally, I provide quantitative guidelines based on total detected heat and conversion of reactants to products that I recommend to replace the c-based guidelines, and I describe a computer program available to facilitate their implementation, requiring only that the user provide estimates of n, K, and ΔH°. Although my emphasis is on statistical aspects of the protocols, in my examples I also discuss how practical considerations (e.g., solubility, aggregation, large blank heats) and systematic error may limit the achievable precision and accuracy.

Computational methods

The calculations employed methods like those used before on this problem [12–14,17,18]. These methods use the predictive properties of the LS a priori covariance matrix $\mathbf{V}_{\text{prior}}$ in experiment design mode by computing the statistical precisions of the key parameters—K, ΔH°, and n—for various assumed values of these and the experimental parameters. This approach requires that $\mathbf{V}_{\text{prior}}$ yield trustworthy estimates of the parameter standard errors (SEs). Because the LS problem here is nonlinear, some have raised questions about this assumption. However, I have shown that $\mathbf{V}_{\text{prior}}$ is reliable for nonlinear LS in application to many common nonlinear data analysis problems by using Monte Carlo (MC) computations to check these predictions [19]. The only condition for this

trustworthiness is that the relative standard error (RSE) be less than ~0.1 (or that the SE be < 10% of the magnitude of the parameter), whereupon the predicted SEs are reliable within 10%. In fact, MC computations on the ITC problem have indicated that the condition RSE < 0.1 is overly demanding under typical circumstances [17,18].

Although it is not necessary to repeat here details of the nonlinear LS computations, it is useful to emphasize several properties of $\mathbf{V}_{\text{prior}}$ [19,20], defined by

$$\mathbf{V}_{\text{prior}} = (\mathbf{X}^T\mathbf{W}\mathbf{X})^{-1}, \tag{2}$$

where the matrix $\mathbf{X}$ contains elements $X_{ij} = (\partial F_i/\partial\beta_j)$, in which F_i represents the fit relation for the ith point, β_j is the jth adjustable parameter (n, $\Delta H°$, and K), and $\mathbf{W}$ is the weight matrix ($m \times m$ for m injections).

- If the data possess random independent error (the usual case), $\mathbf{W}$ is diagonal, with elements $W_{ii} = w_i$. For minimum-variance estimation, the weights must be $w_i = 1/\sigma_i^2$, where σ_i is the standard deviation for the ith point. Then $\mathbf{V}_{\text{prior}}$ is exact for linear LS and reliable within the 10% rule of thumb for nonlinear. "Exact" means, for example, that it correctly predicts the statistical outcome of MC simulations.
- $\mathbf{V}_{\text{prior}}$ is independent of the measured quantities in linear LS but may depend on them in nonlinear LS. Thus, for the latter it is computed using exactly fitting data (e.g., as generated for assumed values of the parameters).
- The parameter variances are the diagonal elements of $\mathbf{V}_{\text{prior}}$; the SEs are the square roots of these. If the data are "normal" (i.e., have random error that is Gaussian about the true values), the parameter estimates will also be normally distributed for linear LS and for nonlinear LS in the limit of small data error. Inherent nonnormality for nonlinear estimators with finite data error is partly responsible for the 10% rule of thumb for nonlinear SEs.
- The parameter variances scale with the data variance, and the SEs scale with σ_i; for example, increasing all σ_i by a factor of 4 will increase all parameter SEs by a factor of 4.
- The off-diagonal elements or covariances yield the correlation coefficients,

$$C_{ij} = \frac{V_{ij}}{(V_{ii}V_{jj})^{1/2}}. \tag{3}$$

  Interparameter correlation is almost always present in both linear and nonlinear LS. Some authors have stated that such correlation renders unreliable the predictions of the covariance matrix, but this is incorrect [21]. Instead, very strong correlation (C_{ij} very close to 1 or −1) signals that σ_i ($=V_{ii}^{1/2}$) is large *because* of this correlation and might be much smaller if additional information could be used to reduce the correlation. A simple example of this is freezing one of two highly correlated parameters at a known value.
- In the analysis of data having random error, the residual δ_i is the difference between measured and calculated values for the ith point. The quantity $S = \sum w_i\,\delta_i^2$ is the target of minimization (hence "least squares"); when $w_i = 1/\sigma_i^2$, S is an estimate of χ^2 and S/ν is the reduced χ^2, χ^2_ν. Here ν is the degrees of statistical freedom (= m − 3 when three parameters are fitted). χ^2_ν has average value 1 and variance $2/\nu$, from which sampling-based estimates of variance have RSE $(2/\nu)^{1/2}$ and estimates of SEs have RSE $(2\nu)^{-1/2}$.
- Scaling $\mathbf{V}_{\text{prior}}$ by S/ν yields the a posteriori version $\mathbf{V}_{\text{post}}$, commonly used in the analysis of actual data when the scale of the data error is not known. Note that this means that $\mathbf{V}_{\text{post}}$ goes to zero for exactly fitting data, whereas $\mathbf{V}_{\text{prior}}$ does not. For unweighted LS, S/ν is an estimate of the data variance σ^2.

Modeling an ITC experiment requires specifying initial concentrations of titrant $[X]_{0,0}$, titrand $[M]_{0,0}$, cell volume V_0, number of injections m, and the ith injection volume v_i. The current computations assume the "instantaneous injection" perfusion model [22], whereby a volume v_i of the equilibrated mixture following the i–1th injection is expelled from the active volume V_0 in the ith injection and the freshly injected titrant then mixes and reacts with the remaining material in V_0. However, such details are not important for producing results like those in Figs. 2 and 3. I have used volume parameters appropriate for the MicroCal VP–ITC—V_0 = 1.4 ml and a total injected volume of 250 μl, the latter being roughly the maximum possible total titrant volume (which is the best choice for very small c, as discussed below).

To compute the weights $w_i = 1/\sigma_i^2$, we need the data variance function. For ITC, this can be approximated as a sum of constant and proportional error terms, a form that has been found to hold for a range of experimental methods. Here I use results obtained for the MicroCal VP–ITC [23],

$$\sigma_i^2 = \sigma_q^2 + (\sigma_p q_i)^2 + (\sigma_v q_i/v_i)^2, \tag{4}$$

with σ_q = 0.8 μcal, σ_p = 0.0024, and σ_v = 0.015 μl. Recent work has supported concerns [23] that this σ_q value is too large; as discussed below, this affects the scale of the computed results but not the qualitative behavior. The value for σ_p is close to estimates for other methods where peak areas are estimated (e.g., high-performance liquid chromatography [24]); it should be roughly independent of σ_q. The last term in Eq. (4) stems from uncertainty in the syringe delivery volume and makes only small contributions to the total variance in the current calculations; it becomes comparable to the second term when v_i < 6.3 μl.

Standard ITC protocol: Fix-ups and false facts

Why increase the titration range?

From Fig. 2, increasing R_m is the key to better results for c < 100. The reason is that with decreasing c, the reaction is progressively less complete for a given R_m (like R_m = 2 in the standard protocol), thereby giving less detected heat and incomplete sampling of the reaction enthalpogram. Eq. (1) was obtained empirically and represents a compromise between complete conversion of M (which would require infinite titrant) and a reasonable fractional conversion f. The latter turns out to be ~93% below c = 1 when Eq. (1) is followed. It can be computed as a function of c and R through the following considerations. For the reaction M + X ⇄ MX, the equilibrium relation is

$$K = \frac{[MX]}{[M][X]} = \frac{[MX]}{([M]_0 - [MX])([X]_0 - [MX])} \tag{5}$$

where $[M]_0$ and $[X]_0$ represent the concentrations prior to reaction. Letting $f_i = [MX]_i/[M]_{0,i}$, where the i subscripts refer to the ith injection, we obtain

$$f_i^2 - f_i(R_i + 1 + 1/c_i) + R_i = 0, \tag{6}$$

which can be solved for f_i for specified R_i and c_i. Fig. 4 illustrates results for $i = m$, with R_m set by Eq. (1), and for several smaller values, including R_m = 2 from the standard protocol.

Use of the titration ranges illustrated with the second and third curves in Fig. 4 leads to increases of ~40 and 70%, respectively, in the RSE for K in the c = 0.002 to 2.0 range. Still, the RSE remains below 3% over this range, which is a sort of practical limit for the method when systematic errors are taken into account (see below). Even decreasing R_m by a factor of 10 achieves better than 12% determination of K, and this loss can often be offset by increasing $[M]_0$. Thus, when practical considerations prevent use of $[X]_0$

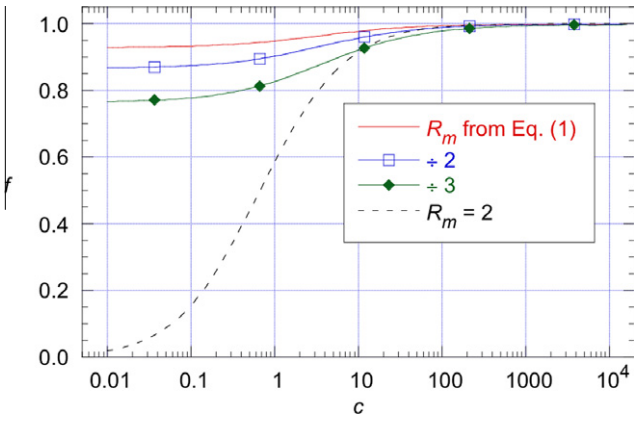

Fig.4. Converted fraction f of titrand M as a function of c for various R_m. For the second and third curves, the altered R_m values were $R'_m = 1 + (R_m - 1)/a$, with $a = 2$ and 3; this ensures that $R'_m > 1$ at large c.

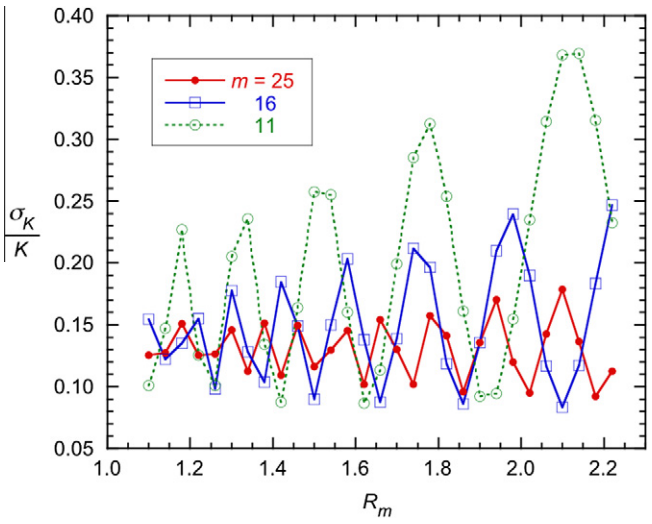

Fig.5. RSE in K for $c = 5000$ as a function of the number m of injections and stoichiometry range R_m. Other parameters are as in Fig. 2.

values as large as given by Eq. (1), users may still achieve success, especially when n is frozen (below).

"Nice idea, but impracticable for biological applications"

This concern stems from the realization that reagents of biological interest are frequently precious, of low solubility, and/or subject to self-association and aggregation. Binding of *two* such materials may be difficult to study with ITC at any c because even in the standard protocol the syringe concentration ($[X]_{0,0}$) must be at least 10 times the cell concentration ($[M]_{0,0}$). More commonly, only one of the reactants is subject to such limitations, and then operating at very low c (with M that reagent) actually serves to minimize such problems. For example, consider a reaction having $K = 10^5\ M^{-1}$ and $\Delta H^\circ = 10$ kcal/mol. By using $[M]_{0,0} = 50\ \mu M$, we get $c = 5$. Then the program described below predicts that we can estimate K with 5% RSE using the standard protocol. But suppose we are limited to $[M]_{0,0} = 5\ \mu M$, giving $c = 0.5$. Then $R_m = 2$ gives an SE $\approx 4\ K$, so ITC fails. Even if we freeze $n = 1$ in the analysis, we still get $\sigma_K \approx K$. On the other hand, if we use Eq. (1), we obtain $R_m = 33$, requiring a syringe concentration of 0.84 mM (vs. ~0.50 mM for the standard protocol with 50 μM titrand), and we can estimate K with 35% RSE. Now if we freeze $n = 1$, this drops to 9% or even lower with variable injection volumes (see below). If we can manage a titrant concentration of only 0.3 mM (giving $R_m = 12$), we can still estimate K with 13% RSE. (All of these SEs drop by a factor of 4 if more optimistic data error is assumed; see below.)

The forgoing example does touch on one limitation in working at low c. As discussed further below, as c decreases below 1, it becomes progressively harder to simultaneously determine n and ΔH° as these parameters become highly correlated. However, both can still be obtained from temperature dependence through either van't Hoff analysis or global analysis of ITC experiments run over a range of temperatures [14].

Why smaller m can be better than larger m

ITC methods articles have sometimes included statements such as, "It is better to use a larger number of smaller volume injections because this gives more points to be fitted." This seems natural but is wrong for ITC, at least in the low-q limit, where $\sigma_i \approx \sigma_q$. The reason is tied to the fact that the starting M in the cell represents a fixed amount of reaction heat, which is subdivided in the titration procedure. The dependence can be understood from the following analogy. Suppose we want to weigh 10 nominally identical coins with a balance that has constant measurement uncertainty $\sigma_m = 0.01$ g. If we weigh them all at once, our uncertainty is 0.01 g; if we weigh them individually and sum, from error propagation the uncertainty is $[10 \times (0.01)^2]^{1/2}$ or 0.032. If we want the average mass, both uncertainties are reduced by the factor 10 and the single measurement remains better.[2]

Now suppose q_i is large enough that σ_i in Eq. (4) is dominated by the second and third terms, making $\sigma_i \propto q_i$. In the mass analogy, take σ_m to be 0.1% of the mass. Letting M be the mass of a single coin, the uncertainty of the collective measurement is $0.01\ M$, whereas for the summed individual measurements it is $[10 \times (0.001\ M)^2]^{1/2} = 0.0032\ M$. The mean uncertainties are again both smaller by a factor of 10; thus, with proportional error (also called constant coefficient of variation), the summed measurement is better.

Because both kinds of statistical error occur in Eq. (4), ITC experiments are optimal with the smallest possible m in the low-q limit but prefer larger m with increasing heat. In fact, the variable-v optimization algorithm developed in Ref. [13] almost always converged to just 3 injections for three fitted parameters, independent of c, when constant data error was assumed. The recommendation for $m = 10$ in Ref. [12] was a balance between the low-q and high-q preferences, giving only modest precision losses from optimal in both cases.

"You need at least p points in the transition region"

This notion is widely accepted and repeated, with p usually ~5. It is ipso facto false from the results shown for $m = 10$ in Fig. 2 and the finding that statistically optimal results are obtained for just 3 injections for most c when the data error is constant [13]. However, the 10-injection protocol is less efficient than the standard protocol above $c = 400$ in Fig. 2, so it is of interest to examine this region more closely. Fig. 5 shows that the precision is indeed sensitive to the *placement* of the injections, but there is no special role played by the transition region and certainly no need for ~5 points because for the 11-injection results for $R_m = 1.1$, only the last 2 q_i differ noticeably from the constant level of the first 9 values. Furthermore, if these last 2 injections (22.73 μl each) are replaced

[2] The uncertainties here are just the measurement uncertainties. If the true coin masses vary by more than this and this variability is of interest, then individual weightings are required.

by 10 injections having the same total volume (4.54 μl each), σ_K *increases* by 25% (after optimizing R_m). Still, from Fig. 5, it is better to use larger m in the very-high-c region if the best precision is needed, because it is not possible to know K well enough to set R_m optimally and there is increased variability in σ_K with smaller m.

In part, this misperception stems from unclear understanding of just what constitutes the "transition region." This ambiguity is obvious for the very-low-c region. In the other extreme, the points just below and just beyond the equivalence point are also, in fact, in the transition region; q values virtually identical to the prior values below equivalence, and identical to subsequent values past equivalence, convey valid information about this region, just as do values that differ in the gentler transition profile for smaller c. Put simply, *all* points define the reaction profile and contribute more or less equally to the determination of the thermodynamic parameters.

"The inverse correlation between K and ΔH° is a major problem in computation"

In MC computations on 10^4 synthetic data sets in a run, the only time I encountered correlation problems (manifested as failed convergence for some data sets) was when c was 0.1 or smaller, and there the problem was the correlation between n and $\Delta H°$ [12]. The difficulty in obtaining converged results was further traced to "reciprocal" behavior of $\Delta H°$ in this region; when the fitted variables were taken as K, n, and $(\Delta H°)^{-1}$, 100% convergence was again achieved.[3] For c in the commonly used range 1 to 100, results presented in Table 1 of Ref. [12] show that of the three interparameter correlations, the one between K and $\Delta H°$ is the smallest in magnitude. For $c < 0.1$, the n–$\Delta H°$ correlation may indeed complicate the fitting. However, there the product $n \times \Delta H°$ is more precise than K, so defining the fit parameters as K, $n \times \Delta H°$, and n ensures success [13].

Some of these dependencies are illustrated in Fig. 6, which also shows how freezing one of two highly correlated parameters greatly reduces the SE for the other (and eliminates the correlation problem in the fitting).[4] If the standard protocol is followed, the correlation between K and $\Delta H°$ may indeed become problematic. For example, it is within 0.001 of −1 for $c = 0.1$ in Fig. 2, closing to within 2×10^{-5} for $c = 0.01$. However, these results are of no practical consequence because the relative error in K exceeds 100% already for $c = 0.25$ in this protocol. Fig. 6 shows further that for $c > 100$, freezing n has little effect on the precisions of K and $\Delta H°$; this is because here n is so well determined in the three-parameter fits that it is already effectively frozen.

The near-independence of K from $\Delta H°$ and n in the very-small-c regime stems from

$$K[\mathrm{X}]_0 \approx \frac{f}{1-f}, \tag{7}$$

obtained from Eq. (5) when $[\mathrm{X}]_0 \gg [\mathrm{M}]_0$, which holds already after the first injection for $c < 0.1$, when R_m is taken from Eq. (1). Thus, the fractional conversion f becomes independent of $[\mathrm{M}]_0$ (and, equivalently, from n for small n). One can fix n at a reasonable value and fit K and $\Delta H°$ with no difficulty. Sizable errors in the adopted n have only a minor effect on the estimated K, so with temperature

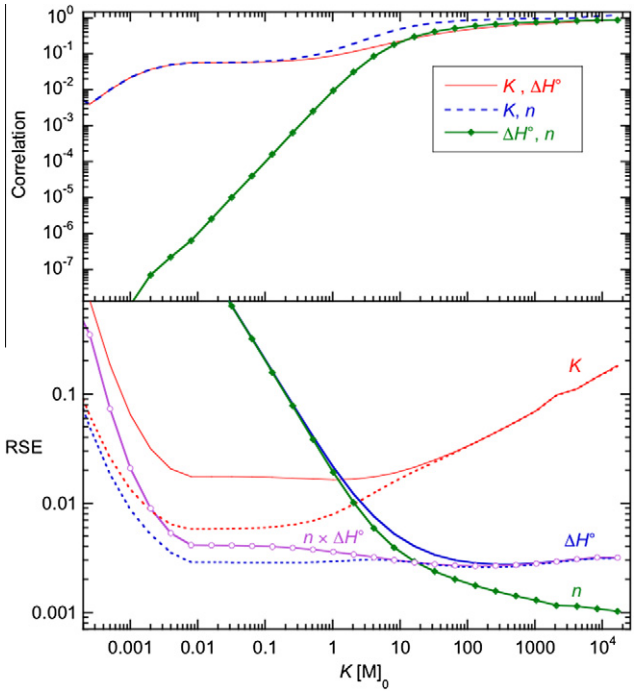

Fig.6. RSE and interparameter correlation versus c for $\Delta H° = 10$ kcal/mol, $[\mathrm{M}]_0 = 0.1$ mM, $m = 25$, and R_m set by Eq. (1). In the lower panel, the curve with open points is for the product of n and $\Delta H°$ when this replaces $\Delta H°$ in the three-parameter fits, whereas the dashed curves represent results from two-parameter fits with n fixed at 1.0. The correlation quantities plotted in the upper panel are $1 - C_{ij}$ for K and n, and $1 + C_{ij}$ for the other two.

dependence for K, $\Delta H°$ can be estimated from a van't Hoff analysis, leading to determination of n and then a refined analysis of K if desired. Alternatively, the T-dependent ITC data can be analyzed simultaneously to achieve optimal results in a single step [25,26].

"The parameters n and ΔH° are linear and, hence, better determined than the nonlinear parameter K"

This claim is related to the preceding one. In the standard protocol, n and $\Delta H°$ are more precisely estimated than K for c in the range 1 to 1000 [13]. However, all three parameters are nonlinear when estimated from a three-parameter LS fit. With R_m set by Eq. (1), K is the most precisely estimated quantity below $c = 2$ (excluding the product $n \times \Delta H°$; see Fig. 6). The most nonlinear parameter is $\Delta H°$ in the low-c region, where it shows reciprocal behavior, as already noted.[5]

In related statements, some authors have stated that n should never be fitted. Although n may need to be frozen in work at very small c, for $c > 1$ there are several reasons why it must be included in the fit model. First, there are situations where the purity or reactive fraction of the titrand is truly unknown. Even when this is not a concern, n is the most precisely determined parameter for $c > 1$, often having $\sigma_n < 0.01$, which is much better than the reliability with which titrand concentrations are commonly known [16]. In addition, n absorbs some of the errors associated with uncertainty in the active volume V_0 [2]. Thus, freezing it at its presumed known value can introduce sizable systematic errors into the estimates of K and $\Delta H°$. On the other hand, fitted values of n far from expected should rightly be seen as a warning of experimental problems.

[3] Convergence problems in nonlinear LS can arise from strong correlation but also from highly nonnormal parameters—especially those that do not have finite variance such as reciprocals of normal parameters—and are exacerbated by large data error [19]. Hansen and coworkers [8] reported convergence problems in their MC computations, but their relevant data errors were large, giving $\sigma_K \geqslant K$ in the extreme. Their results for $\sigma_q = 1\%$ of q_1 are commensurate with those obtained here for the standard protocol when allowance is made for their fitting of just K and $\Delta H°$ and their variable σ_q (given that q_1 varies with c).

[4] The plotted correlation properties are the logarithmic closeness of the C_{ij} to 1 for positive correlation, and to −1 for negative correlation, because these quantities best predict LS fitting problems.

[5] $\Delta H°$ is linear in a one-parameter fit with K and n frozen. Even though highly correlated with $\Delta H°$ and very uncertain at low c, n remains close to normal in MC simulations [14].

"Because n compensates for errors in the active volume V_0, there is no need to know V_0"

This is, of course, not true if actual values of n (i.e., purity or reactive fraction) are of interest. Furthermore, although V_0 errors do not affect the ΔH° estimates in the standard analysis algorithms, they do affect K and in a proportionate way. Thus, if V_0 requires correction by the factor 0.94, as was found in Ref. [22] for a VP–ITC, so does K; that is, lowering V_0 by 6% will also lower the estimated K for any data set by 6%. This is a significant systematic error in many cases, so it is advisable to carry out a determination of V_0. The NaCl dilution method of Ref. [22] works well to determine both V_0 and the heat correction; however, the relative apparent molar enthalpy function for NaCl (aq) is very sensitive to temperature, giving a change of > 1% in V_0 for just a 0.12-K error at 25 °C. And this T error is well within the stated 0.2-K reliability for the VP–ITC. Thus, without accurate T calibration, it is better to use a simple reaction with easily handled, well-behaved reagents. The Ba^{2+} + 18-crown-6 ether complexation in water is useful for this purpose and is recommended by one instrument maker.[6]

"ΔH° can be estimated from the early-injection heat, where titrant is fully converted"

This point is relevant for the situation where only ΔH° is needed. Considering Eq. (5) in the limit $[M]_0 \gg [X]_0$, we have

$$K[M]_0 = c \approx \frac{g}{1-g}, \tag{8}$$

where g represents the converted fraction of titrant, $[MX]/[X]_0$. If $c \approx 20$ or greater, $g \approx 1$ and ΔH° can be estimated from $q_1 \approx \Delta H^\circ V_0 [X]_{0,1} = \Delta H^\circ v_1 [X]_{0,0}$. On the other hand, if $c < 0.1$, we have $g \approx c$, giving

$$\Delta H^\circ \approx \frac{q_1}{v_1 [X]_{0,0} c}, \tag{9}$$

requiring knowledge of K for estimation of ΔH°.

"ITC is fundamentally a technique for measuring differential heat"

Wiseman and coworkers [1] obtained an expression for $dq/d[X]_0$ and implemented it with finite differences in their data analysis. This approach was followed by Bundle and Sigurskjold [3], by Indyk and Fisher [27], and very recently by Poon [28], who also proposed fitting the integrated differential equation directly. The differential treatment may be a historical consequence of the earlier development of instruments that did record differential heat [8], and it might be applicable to data collected with the "single-injection method" [29]. But for standard use of modern titration calorimeters as treated here, the differential picture seems doubtful just from the description of each step of the experiment: An aliquot of titrant is injected, it mixes and reacts with the material in the cell to produce a peak in the enthalpogram, and the power returns to baseline after several minutes. Integration of the peak yields the heat q, which is related to the before-and-after changes in the composition of the cell mixture. Nothing about this procedure requires constant-v injections, so finite differences *must* to be considered in any attempt to use a differential treatment for variable v_i.

Although a constant-v, large-m protocol must approximate the differential treatment, the integral treatment, as employed at least by 1994 [30], seems to be directly applicable to any sequential injection protocol, including variable-v schemes. Still, there do remain questions about the mixing of reagents in the cell and the definition of the active volume [13,22]. The treatment of Ref. [30] corresponds to an assumption of instantaneous injection followed by mixing, whereas the Origin algorithm used by most who work with MicroCal instruments approximates instantaneous mixing. However, the Origin program has not always been completely consistent with this model, according to which the total concentrations of the reagents in the cell after the ith injection should be [22]

$$[X]_{0,i} = [X]_{0,0}\left[1 - \exp\left(-\frac{v_{\mathrm{cum},i}}{V_0}\right)\right] \tag{10}$$

and

$$[M]_{0,i} = [M]_{0,0} \exp\left(-\frac{v_{\mathrm{cum},i}}{V_0}\right), \tag{11}$$

where $v_{\mathrm{cum},i}$ is the cumulative injection volume and the 0,0 subscripts again represent the prepared solution concentrations.[7]

From inspection of raw ITC data, it is clear that the instant mixing model is far from correct. Thus, for example, the inset to Fig. 4 in Ref. [22] shows clear mixing-related oscillations that last for > 30 s after a 10-s injection, with > 100 s required for return to baseline. In Ref. [13], where a variable-v, 3-injection protocol was used, the instant injection model was better than the instant mixing model. However, for the very long-duration third injection (~200 s), mixing did seem to contribute to some loss of newly injected titrant.

To check the sensitivity of results to this choice of mixing model, I have generated synthetic data assuming instantaneous injection and then analyzed them using both versions of the Origin algorithm (see note 7). For 25 10-μl injections, the differences in K and $n \times \Delta H^\circ$ are ~ 0.3% for $c = 10^{-3}$ to 10^4, increasing to ~ 7% for K at both extremes when the Origin algorithm is used (Fig. 7). The differences for 10 25-μl injections are larger but roughly track those in Fig. 7, remaining within ~ 1% for $c = 10^{-3}$ to 10^3 when the corrected concentrations are used. The differences for n and ΔH° (not shown) become large for $c < 0.1$ but are still within the large statistical errors in these quantities in this region. All of the differences are greatest for the largest possible $v_{\mathrm{cum},m}$, which is roughly true for the 250 μl used in these calculations.

"Errors in concentration and baseline increasingly affect K and ΔH° as c decreases below 50"

Effects of baseline error can be checked by adding a constant to synthetic q_i data, refitting, and comparing the altered values of the parameters with their initial values. Fig. 8 illustrates results of this exercise for simulated data like those used to prepare Figs. 2 and 6. The assumed baseline error amounts to < 2% of q_{tot} but does produce significant errors in K and ΔH°—approximately 6% (negative) and 2%, respectively, over much of the displayed range of c. Both systematic errors do increase below $c = 10$ in the standard protocol, but they remain roughly constant when R_m is set by Eq. (1), increasing only when this relation gives way to the adopted limit on $[X]_{0,0}$. With n frozen at 1.0, the effects of this systematic error are actually lowest in the low-c region, 0.004 to 1 (where often n must be fixed for analysis). When n is fitted, it is the most sensitive to baseline errors below $c = 0.3$.

As has been discussed recently, concentration errors have exactly predictable effects on the estimated values of all three parameters, and these effects are independent of c [16]. In the

[6] That manufacturer is Calorimetry Sciences Corp. (now part of TA Instruments). The V_0 correction factor found this way in Ref. [2] was 0.97; however, this determination was made before discovery of the syringe volume delivery error of 37/38 discussed in Ref. [22].

[7] The original Origin algorithm corresponds to truncating the Taylor series expansion for the exponential after three terms. The expression for the heat q_i can be obtained by approximating an integral as the product of the average integrand and v_i, as described in Ref. [22]. Recent versions of the software are said to now employ Eqs. (10) and (11).

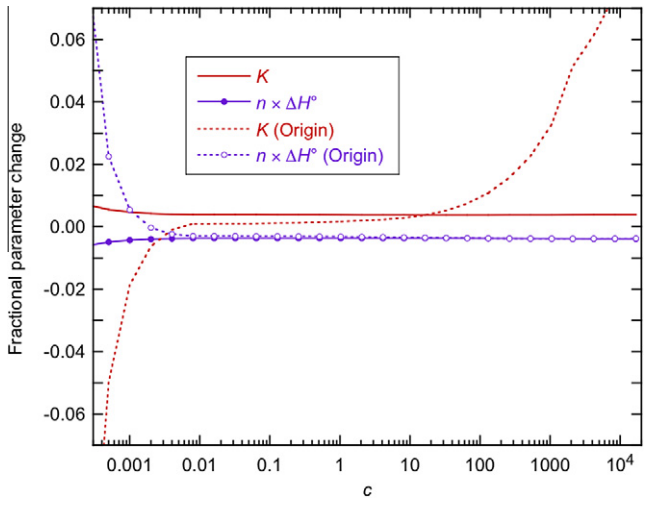

Fig.7. Dependence of estimated K and $n \times \Delta H^\circ$ on mixing model. Plotted quantities are the relative changes in these parameters when fitted with the instantaneous mixing model versus the instantaneous injection model. Dashed curves and open points represent results obtained using the approximations in the original Origin algorithm.

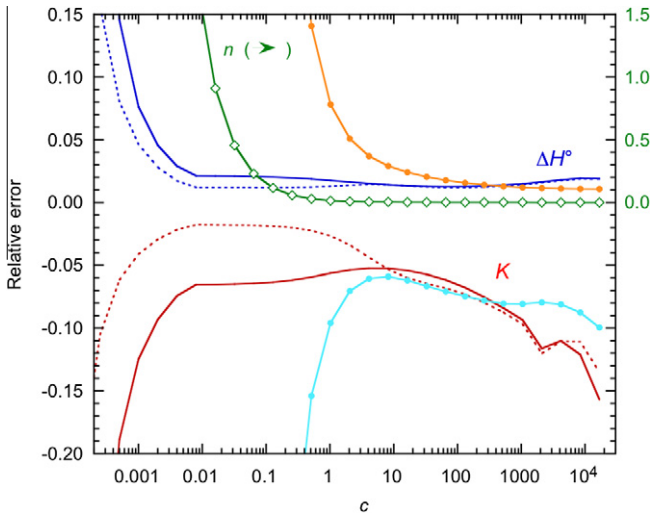

Fig.8. Effects of baseline error, for synthetic data like those used to prepare Figs. 2 and 6, with 1 μcal added to each of 25 q_i. The plotted relative errors are defined in the sense, (altered – true)/true, and are negative for K and positive for ΔH° and n. Solid curves represent results obtained by fitting all three parameters, whereas dashed curves are for n fixed at 1. Curves with solid points were obtained using $R_m = 2$ and others with R_m defined by Eq. (1). Note the different y-axis scale at the right for n (open points).

standard analysis algorithms, the titrant concentration is treated as exact, so any uncertainty there needs to be taken into account in the reporting of the SEs. A quantitative summary of the effects of systematic errors is given in Table 1 of Ref. [12].

"Always include a small throwaway first injection in your protocol"

This procedure was devised to counter the observation that the heat for the first injection was consistently low in magnitude, explained as due to diffusive loss of titrant from the syringe during the long preinjection equilibration period. However, it has been shown that the heat shortfall is mainly due to backlash in the screw mechanism that drives the plunger in the syringe, an effect that leads to a shortfall of ~ 1 μl in the delivered volume of titrant from the first injection [31]. The problem arises because for the user following standard procedures, the first injection produces the first down motion of the plunger following an up motion at the end of the filling procedure. If, instead, a down motion is executed before the syringe is installed in the cell, the diffusive loss drops to < 0.1 μl.

The diffusive loss of 0.1 μl (sometimes more) can still represent a significant heat shortfall for the first injection, so a small, discardable first injection may still be wise. In any event, the prior down motion must be included. Without it, there is a ~ 1-μl discrepancy between actual and assumed volume of titrant, which propagates through the computed concentrations $[X]_{0,i}$ and $[M]_{0,i}$ for all injections. The effect of this error will be greatest for the smallest total titrant volume $v_{\mathrm{cum},m}$ and can be estimated by considering the discrepancy to the stoichiometric endpoint. For example, with 10 10-μl injections to $R_m = 2$, the discrepancy at the endpoint ($R_m = 1$) is 2%, and this results in a 2% overshoot in both n and K. With the use of Eq. (1) to set R_m, the error can be larger because the endpoint is reached sooner in the titration; thus, for $c < 1$, the errors in n and K can exceed 10% for 10 10-μl injections. On the other hand, for ΔH°, detailed computations show that such backlash-caused errors are negligible under all circumstances.

What is the data error in ITC, and what difference does it make?

Any attempt to predict the precision of estimation of the parameters from an ITC experiment, whether from MC simulations [6,8,10,26] or from $\mathbf{V}_{\mathrm{prior}}$, requires some assumption about the data error. Workers who analyze their data using the Origin software supplied with MicroCal instruments by default employ unweighted nonlinear LS, which tacitly assumes constant data error.[8] In the typical unweighted analysis, the quantity S/ν defined above becomes an estimate of the data variance and its square root the data σ. MC simulations that employ constant data error of this magnitude will confirm the SE results obtained from these LS analyses provided that the parameter RSEs do not significantly exceed 10%.[9] On the other hand, if σ_i varies with i (e.g., for error proportional to q), weighted LS is required for correct analysis, including in MC simulations for such data.

Because the variance function of Eq. (4) does depend on q, weighted LS is required for correct analysis. However, the analysis that yielded this expression was based on data having relatively large q_i, > 2 mcal for some early injections. For so much heat, the proportional error terms in the variance function of Eq. (4) dominate, making the analysis sensitive to these terms. For comparison, in the computations behind Figs. 2 and 6, the maximum q_i is < 0.8 mcal, giving σ_i that vary by at most a factor of 2.5. When the data errors span a range this small, their neglect in the LS analysis generally has only a modest effect on the results. Furthermore, the RSE in K is already < 0.02, where systematic errors like those just discussed—volume uncertainty, fit model limitations, baseline error, and concentration uncertainty—likely limit the reliability of the determination.

Systematic errors will normally remain limiting for reactions with more heat, where weighting should be more important and the SEs smaller. Thus, unless great effort is made to address

[8] Weighted fitting can be done with the Origin package, as discussed in Ref. [12] (p. 20034). Note that the quantity actually fitted in the Origin program is called "NDH" and is obtained from the heat q ("DH") by dividing q_i by $\Delta n_{X,i} = v_i [X]_{0,0}$. Accordingly, the data uncertainty σ_q must be backed out of the output from the fit. For example, a particular Origin fit yielded "Chi^2/DoF = 3447," from which $\sigma_{\mathrm{NDH}} = (3447)^{1/2}$ cal/mol. The experiment involved 10-μl injections of 0.72 mM titrant, yielding $\sigma_q = 0.42$ μcal.

[9] The statistical properties of χ^2 limit the precision of SEs from MC simulations. For example, the $(2\nu)^{-1/2}$ RSE in the SE means that SEs from 100 MC simulations are uncertain by 7%. For defining confidence limits, the variability can be even greater from the small number of values that define the wings (e.g., just 10 for 90% confidence from 100 simulations). Similar limitations must hold for MC methods that estimate confidences from the properties of SSR (sum of squared residuals) for single data sets, as in Ref. [10].

systematic effects, the extra statistical precision will be a misleading indicator of the true reliability. As an example of this, Table 1 in Ref. [13] gives several K and ΔH° values from experiments done by me at high and low q. The former yielded RSEs < 0.01, but the actual values changed by $\sim 4\ \sigma$ just from using updated weights based on Eq. (4) and calibration results for V_0 and power [22]. The low-q results were much less precise but also showed variability greatly exceeding their SEs, tentatively attributed to dilution heat problems not accommodated in the fit model. From such considerations, it seems likely that $\sim 3\%$ is a practical limit for the current capabilities of ITC in all but the most painstaking experimental work. And this precision level can be usually achieved with q_i values small enough to make σ_i^2 nearly constant and render weighting unimportant.[10]

In the low-q limit, the predicted parameter SEs scale with σ_q in Eq. (1). The Ref. [23] value of 0.77 μcal (unrounded) was based on data sets that had blanks subtracted before analysis, so the single-measurement σ_q is smaller by $2^{1/2}$ or $\sigma_q \approx 0.5$ μcal. Furthermore, the data were collected using the least sensitive scale of the instrument, with most q_i in the range 200 to 2000 μcal. Recent work with small q_i measured from enthalpograms recorded with the most sensitive scale of the VP–ITC has yielded $\sigma_q \leqslant 0.2$ μcal, and possibly as small as 0.1 μcal, when improved procedures are used to estimate the baseline (S. E. Boyce et al., unpublished). Lowering σ_q to 0.2 μcal lowers all of the curves in Figs. 2, 3, 5 and 6 by a factor of ~ 4. Although it also lowers the q level where proportional error becomes significant, the same considerations about systematic errors still limit the need for weighted analysis to those cases where systematic errors can, with confidence, be lowered below the 3% level.

Changing the heat content of the reaction (h) also has a proportionate effect on the RSEs in the opposite direction. Thus, if the concentration $[M]_0$ is lowered to 10 μM, the curves in the lower panel of Fig. 6 are shifted up a factor of 10, whereas decreasing σ_q to 0.2 μcal drops them a factor of 4, for a net increase by a factor of 2.5. Accordingly, an ITC experiment can yield K with 10% precision for c in the range 0.0005 to 200, or $K = 50 - 2 \times 10^7\ M^{-1}$, needing just 15 nmol of the cell reagent. This result also depends on ΔH° given that h is the product of ΔH° and $[M]_0$.

Variable-v protocols

For binding processes at very low c, it is advantageous to employ variable injection volumes. The reason is that for heat profiles like that shown in Fig. 1 for $c = 0.003$, a constant-v_i scheme yields most of the total heat in the first few injections and relatively little in all subsequent injections. Thus, by increasing v_i with i, one can redistribute the heat into later injections. At first guess, a scheme with roughly equal q_i for all i might seem ideal. SE computations show that this distribution is not optimal; however, the results are not very sensitive to exactly how the heat is so distributed.

In Ref. [13], I found that in the low-q limit, complete optimization with respect to v_i always led to several v_i going to zero until $m = 3$ (for three fitted parameters) or $m = 2$ (n frozen). Because there is no margin of safety against bad data when all points are fitted exactly, I employed an exponential construct for v_i to design schemes having more than the minimal number of injections,

$$v_i = C \exp(b \times i), \tag{12}$$

with the constant C adjusted to make the sum of the v_i equal to the total titrant volume. I used this approach to obtain schemes having 2 statistical degrees of freedom—4 injections for n frozen and 5 when n is fitted. These protocols give precision close to optimal

[10] This statement requires qualification for $c > 100$, where more total heat is required to achieve 3% precision in K, making weighting potentially more important.

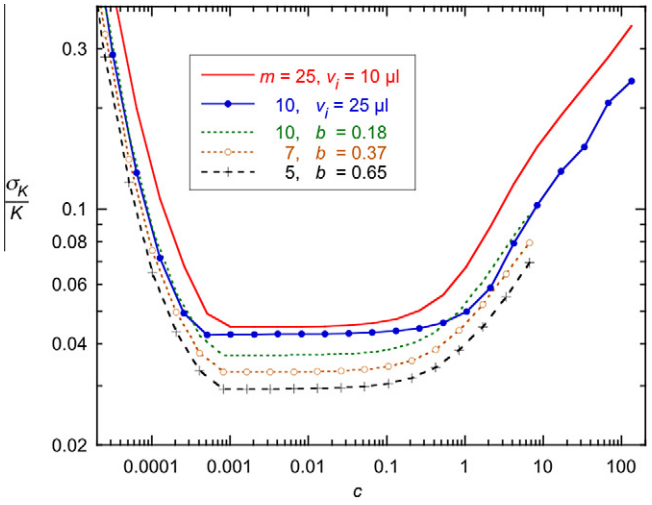

Fig.9. RSE in K as a function of c for various titrant volume schemes with n frozen at 1. The three curves with stated values of b use volumes defined by Eqs. (12) and (13). Other parameters: $\Delta H^\circ = 10$ kcal/mol, $[M]_0 = 10$ μM, $V_0 = 1.4$ ml, and $v_{\mathrm{cum},m} = 250$ μl. Below $c = 0.001$, the upturns in all curves result from the limiting value 1 M placed on the titrant concentration, which thus limits R_m. In all cases, the RSE in ΔH° (not shown) is approximately half that in K across this c range.

and should be useful in cases where the ITC method is being pushed to its limits. However, when the situation is not so marginal, most workers would prefer > 2 degrees of freedom, so I have pursued this matter further. For n frozen and $m = 5$ to 10, I find that the optimal b in Eq. (12) can be well represented by

$$b = \frac{4.5}{m} - 0.27. \tag{13}$$

Some low-h results for variable v_i and fixed n are compared with those for constant v_i in Fig. 9. Several comments are in order. First, consistent with results in Figs. 2 and 6, when R_m is adjusted to compensate for decreasing product conversion, the optimal region is $c < 1$, limited on the small-c end only by the need for large titrant concentrations. Second, the gains come both from v_i optimization and from decreased number of injections, with variable v_i relatively more important for small m. Third, before using these results, workers should be confident that their reaction is truly 1:1 given that 5 to 10 injections cannot provide much information about deviation from this model; however, anomalously large χ^2 values (or large data σ values in unweighted fitting) should still warn of such problems. Fourth, as before, these results were computed using $\sigma_q = 0.8$ μcal; if this is lowered to 0.2 μcal, as seems reasonable (see above), the computed precisions are similarly lowered. Thus, for example, these results would apply for $\Delta H^\circ = 10$ kcal/mol and $[M]_0 = 2.5$ μM or 1 kcal/mol and 25 μM.

All of the forgoing has been premised on the use of Eq. (1) to achieve high fractional product conversion, $f \approx 0.9$. If practical considerations limit R_m and $[X]_{0,0}$ to values much smaller than this prescription, successful results can still be achieved by, for example, increasing $[M]_0$ to get more reaction heat (see below). Under such circumstances, variable-v procedures help little and users can stick with the constant-v schemes.

New guidelines for ITC

From the forgoing, the main requirements for successful use of ITC are (i) sufficient total heat q_{tot} and (ii) adequate conversion of titrand to product. For the curves in Figs. 2 and 6, q_{tot} is ~ 1300 μcal, with product conversion from 100% for large c to 93% at small c (before the low-c rise in RSE caused by the limit placed on $[X]_{0,0}$).

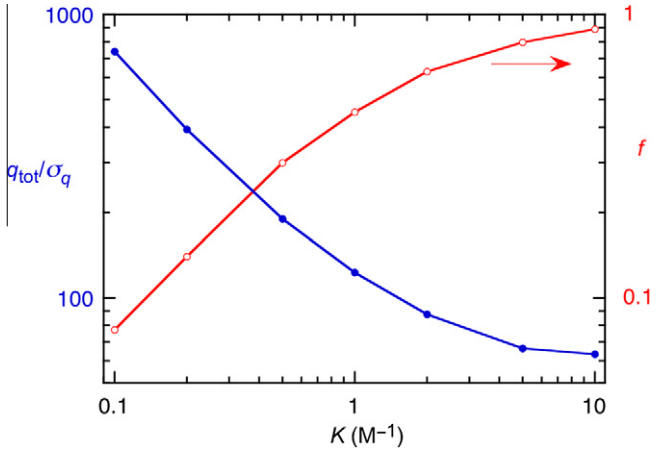

Fig.10. q_{tot}/σ_q needed to achieve 10% RSE in K for $K = 0.1$ to 10 M^{-1} when $[X]_{0,0} = 5$ M, the $V_0/v_{cum,m}$ ratio is 5.6, and n is frozen in the analysis. Corresponding fractional conversions f are included (scale to right). Over this range of K, the RSE in ΔH° declines from 9 to 5%.

Because the RSEs scale approximately with σ_q at low q, it is actually q_{tot}/σ_q that determines the RSE. Thus, K can be obtained with 5% precision for $c = 0.002$ to 100 when this ratio is 700 and all three parameters are fitted. For $c > 100$, this ratio must be increased progressively, to ~ 5000 at $c = 5000$, to maintain this 5% precision. The heats relevant to the curves in Fig. 8 are a factor of 10 lower, and with n frozen we can achieve 5% precision for K with $q_{tot}/\sigma_q = 150$ for 25 injections, dropping to 100 for 5 injections but over a slightly reduced range of c (<1). Again, increasing c requires this ratio to increase until it matches that for fitting all three parameters when $c > 40$.

There is essentially no statistical limit to these results on the small-c end, but there is a practical limit from the need for large $[X]_{0,0}$ to achieve sufficient conversion of M to MX. Thus, from Eq. (6), for 90% conversion at low c, we require $[X]_0 \approx 10/K$ independent of $[M]_0$. For a typical cell/syringe volume ratio of 5, this means $[X]_{0,0} \approx 50/K$, apparently requiring $K > 10$ M^{-1} and much more than this for many biochemical processes, where 5 M titrant is unrealistic. However, as noted above, it may be possible to offset the losses from reduced fractional conversion f by increasing $[M]_0$ to obtain more heat. Fig. 10 illustrates the q_{tot} demand for achieving 10% estimation of K down to $K = 0.1$ M^{-1}, and the corresponding reduction in f, for an initial titrant concentration of 5 M, close to the maximum possible under any circumstances. The computations involved various $[M]_{0,0}$ and ΔH° values, but for reference the results for $K = 0.1$ M^{-1} were obtained using $[M]_{0,0} = 2$ mM and $\Delta H^\circ = 3$ kcal/mol. This precision could also be obtained by coupling somewhat smaller $[X]_{0,0}$ with larger values of $[M]_{0,0}$ and/or ΔH°. For example, $[X]_{0,0} = 2$ M, $[M]_{0,0} = 5$ mM, and $\Delta H^\circ = 7.5$ kcal/mol also yield 10% estimation of K (see supplementary material for illustration of this case). However, the converted fraction is then only 3.2%, and the heats (for $m = 10$) vary only from 185 to 125 μcal, placing great demands on establishing the baseline from dilution blank measurements. Furthermore, large $[X]_{0,0}$ likely means large blank heats [13].

At the large-c end we do encounter statistical limits, as in Fig. 3. If we take $c = 10^4$ as this limit and use $q_{tot}/\sigma_q = 5000$ (for 5% RSE in K) with $\sigma_q = 0.2$ μcal, we need $q_{tot} = 1$ mcal; with $V_0 = 1$ ml, we obtain $K \approx 10^4$ L/cal ΔH°, giving an absolute maximum K of 10^8 M^{-1} for $\Delta H^\circ = 10$ kcal/mol. Under conditions where K can be estimated within 5%, ΔH° (or $n \times \Delta H^\circ$ below $c = 10$ if n is fitted) is typically determined with greater precision. ΔH° can also be determined reliably from single-injection heats for $c > 10^3$.

A computer application for simulating ITC experiments

The program, ITC–PLANNER, can be used to generate results like those discussed here. It follows the perfusion model described above and starts with assumed values for the variance function parameters in Eq. (4) and values for V_0, m, and $v_{cum,m}$; users are prompted to change any or all of these if they desire. The program may be directed to fit all three key parameters or just K and ΔH° with n frozen; in the former case, the user may choose to fit $n \times \Delta H^\circ$ in place of ΔH°. Injection volumes may be of constant v or variable v_i including the exponential distribution of Eqs. (12) and (13). The user enters $[M]_{0,0}$ and then n, K, and ΔH° in response to prompts. R_m and $[X]_{0,0}$ are computed using Eq. (1), but the user is given the option of changing $[X]_{0,0}$. Some results are printed in the working window, whereas complete results—concentrations, parameters and their SEs, $\mathbf{V}_{prior}$ and correlation matrices, and computed values of v_i, $[X]_{0,i}$, $[M]_{0,i}$, $[MX]_i$, f_i, q_i, σ_i, and w_i for each of the m injections—are written to a file named ITC–PRED.LPT, whereas key results are appended to a file named ITC–PRED.DAT.

The program is available in the supplementary material, where samples of the run window and the LPT file are also included. There are two versions: one that is thought to run successfully on all 32-bit DOS/Windows computers and a second that runs on 32- and 64-bit machines in a command-prompt window. The LPT file is overwritten with each execution of the program, so it must be renamed for preservation; the DAT file is an accumulation of single records giving key results from all runs executed in the folder where it resides.

Although there are a number of ITC simulation programs available for use, such as those from the instrument manufacturers and others [7,10,26], I am unaware of any that facilitate experiment design as straightforwardly as this one, with reliable prediction of parameter precisions based on realistic estimates of the data error. The starting parameters in the program are conservative for the VP–ITC, as has already been noted. Workers using other instruments will need to know the approximate data error in the heats q obtained from those instruments.

Conclusion

The "standard protocol" for ITC 1:1 binding experiments calls for the use of ~ 25 injections of titrant to a final titrant/titrand ratio R_m of ~ 2. The latter is a major and unnecessary limiting factor for the applicability of ITC. With it, the method is restricted to values of $c = K[M]_0$ in the approximate range 1 to 5000. However, when R_m is varied to ensure more complete conversion of titrand M to product, the precision for estimation of the binding constant K increases with decreasing c to $c \approx 1$ and then levels off, increasing at much smaller c only from practical limits on the maximum concentration $[X]_{0,0}$ of titrant in the syringe, which in turns limits R_m. There is also no need for 25 injections; the 10-injection programs yield comparable results over the full range of usable c, and for very low c and low h ($=\Delta H^\circ [M]_0$), even fewer injections are preferable. These results directly refute claims of a need for ~ 5 points in the "transition region" of the enthalpogram. Literature statements about the nonlinearity and intercorrelation of the ITC parameters in nonlinear LS analysis are also confronted, as are questions about the nature of the experiment itself (differential or integral), the role of baseline error, and the treatment of injection and mixing in the cell.

In principle, ITC can be used to characterize 1:1 binding processes with precisions better than 1% for K and ΔH°. However, a more realistic limit is ~ 3% when systematic errors are considered. In the MIRG (Molecular Interactions Research Group) interlaboratory study of Ref. [15], the overall variability of the key parameters exceeded their individual SEs by factors of 6 to 16. Most of this

variability has been attributed to concentration errors on the order of 10% [16]. With care, such preparation errors should be reducible to 1% for at least one of the two reagents. However, concerns about the active cell volume and the nature of the mixing in perfusion instruments cannot be so easily resolved. This matter is increasingly problematic when larger total injection volumes are employed, especially in small-*m* protocols. For example, in the study of the benchmark Ba^{2+}/crown ether complexation reaction in Ref. [13], estimates of K and ΔH° varied by 16 and 4%, respectively, depending on how the mixing was treated for a large (196-μl) injection (Fig. 11 in Ref. [13]). This problem might be solved by running experiments with incompletely filled cells, such that all material remains in the active volume throughout the experiment. However, there are concerns about the linearity and accuracy of power detection for a cell containing a variable volume of liquid, requiring careful calibration experiments.

For most biochemical and biophysical applications, the systematic uncertainties are sufficiently small that ITC remains a powerful technique for studying binding. Then, whether a specific process can be studied by ITC requires the answers to two questions. First, can I put enough M in the cell to get adequate reaction heat? Second, can I put enough titrant X in the syringe to realize that heat through conversion of M to the complex MX? Practical and statistical considerations indicate that these questions can be answered satisfactorily to determine K and ΔH° with < 5% uncertainty when K is in the range 10 to 10^8 M^{-1}, extending down to 0.1 M^{-1} for sufficiently energetic binding processes, provided the needed large titrant concentrations are within solubility limits and their dilution heats can be reliably estimated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.12.035.

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