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Optimizing Experimental Parameters in Isothermal Titration Calorimetry: Variable Volume Procedures

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In the study of 1:1 binding, M + X ⇄ MX, isothermal titration calorimetry is generally thought to be limited to reactions in which the key parameter, $c = K[M]_0$, can be set in the range 1−1000. In fact, the range of applicability can be extended by a factor of 10−100 at the upper end and as much as 10^5 at the lower, with certain provisos. The present work emphasizes the low-c regime, with the key heat parameter, $h \equiv \Delta H^\circ[M]_0$, low, as well. Successful determination of K and ΔH° in this region requires that the titration be extended to large excesses of titrant X over titrate M, and then the reaction heat is distributed strongly in favor of the early injections. With decreasing c, ΔH° and the stoichiometry parameter n (often called site number) also become highly correlated and individually indeterminate. However, the product $\Delta H^\circ \times n$ ($\equiv H_n$) is well-determined, so if n is known from other information, both K and ΔH° can be determined to quite low c. By varying the titrant volume from injection to injection, one can significantly reduce the uncertainties in the estimated K and H_n values, permitting determination of K to better than 10% and H_n within 3% down to $c = 10^{-4}$, even for the low h value of 0.1 cal/L. The titrant volume optimization algorithm yields best results for the minimal number of injections — three when n is fitted, two when it is fixed. At low c, the resulting volume distributions depend nearly exponentially on injection number. This observation facilitates the derivation of similar, near-optimal volume distributions for five- and four-injection procedures that offer two statistical degrees of freedom for analysis. The volume optimization results are tested on the Ba^{2+}/18-crown-6 ether complexation reaction at $c = 0.1$ and $h = 0.16$ cal/L, illustrating some practical complications but confirming the utility of the variable-volume protocol.

In isothermal titration calorimetry (ITC), one reactant (titrant X) is injected into a cell containing the other reactant (titrate M) in sequential fashion, and the heat of reaction is measured for each of the typically 10−30 injections. Taken as a function of the extent of reaction, this heat constitutes a titration curve, the analysis of which yields the enthalpy change, ΔH°, and the equilibrium binding constant, K, for the reaction. In simplest terms, K is determined from the *shape* of this curve, while ΔH° is determined by its *scale*. Closer examination shows that the shape of the curve is determined by the parameter $c \equiv K[M]_0$,[1] and the scale, by $h \equiv \Delta H^\circ[M]_0$,[2] where $[M]_0$ is the initial concentration of titrate in the cell. Titration curves spanning the extreme range of c over which ITC is considered applicable are illustrated in Figure 1 for the simplest case of 1:1 binding, to which the present work is confined.

With currently available instrumentation,[1,3] it is possible to estimate both K and ΔH° with relative standard errors less than 1% over much of the range of c illustrated in Figure 1.[2] However, that requires favorable circumstances, especially a value of $h > 1$ cal/L. For heat-starved reactions and titrate reagents that are limited in availability or solubility, this demand may be hard to meet. In the present work, the focus is on optimal design of ITC experiments for such less-than-ideal reactions through the use of nonconstant titrant injection volumes.

ITC is thought to be at its best when applied to reactions such as that illustrated for $c = 30$ in Figure 1. Typical procedures involve titrating to about 2 equivalents of titrant with ∼25 injections. Several studies[2,4−7] have shown that this procedure is not optimal for two reasons: (1) 25 injections is usually too many, and (2) the titration range often should be much greater than 2 equivalents. From a study of the least-squares (LS) statistical errors in the parameters as functions of c, h, titration range, and number of injections,[2] I found that $m = 10$ injections is close to optimal under most conditions and that the titration range should be set by

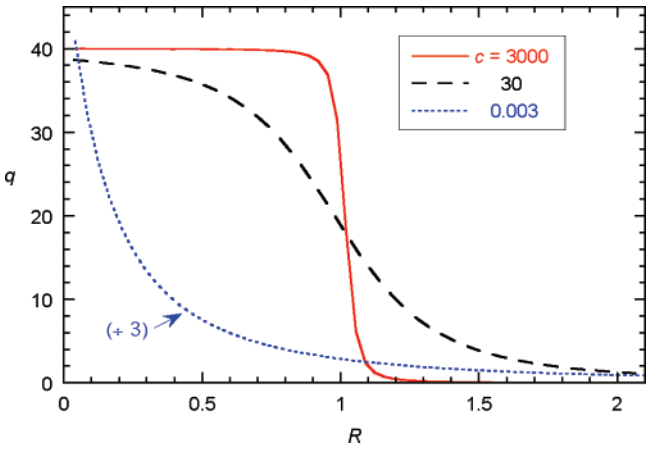

Figure 1. Heat as a function of titration range for 1:1 binding and several values of $c \equiv K[M]_0$. The units for q are arbitrary, but have been adjusted to make the total heat for complete reaction the same in the three cases. For $c = 0.003$, the heat is divided by 3 and the range of R is compressed by a factor of 1000.

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$$R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c} \quad (1)$$

where R_m is the ratio of total titrant to total titrate, $[X]_0/[M]_0$, in the cell after the last (mth) injection. This procedure, of course, assumes that the reaction under study is 1:1 binding, and it requires at least approximate knowledge of K in advance. The performance of this protocol is compared with that for the common default procedure in Figure 2, showing that for very low reaction heat, the default scheme achieves 40% relative standard error (RSE) in K for only a narrow range of c. By contrast, the 10-injection scheme yields RSE < 20% for K down to $c \approx 10^{-4}$. Unfortunately, $\Delta H°$ and the stoichiometry parameter n become strongly correlated below $c = 2$, and it is impossible to determine them individually there without other information about the system under study. However, the product $\Delta H° \times n$ ($\equiv H_n$) is even better determined than K, so when n is well-known from other information, $\Delta H°$ is reliably determined. (Indeed, in many methods for determining binding constants, a stoichiometry parameter is not even considered, because solution concentrations are assumed to be known with confidence.[8])

The 10-injection protocol is still within the standard framework of constant titrant volume for all injections. It is reasonable to ask if varying the injection volume, v, during the titration program might yield further improvement. Consider, for example, the curve for $c = 0.003$ in Figure 1. Equation 1 calls for an R_m ratio >4000, and the use of constant titrant volumes would give most of the heat in the first one or two injections. Indeed, it turns out that the use of small volume v for early injections and large v for later ones can increase the precision with which K and H_n are determined at small c by as much as a factor of ~5. This means that a single experiment done optimally can be the equivalent of 25 done with constant v, which in turn means that many reactions previously thought not to be amenable to study by ITC can, indeed, be well-characterized with this method.

In subsequent sections, I review the statistical methods used to determine the parameter precisions and then describe an algorithm devised to vary the injection volumes systematically in a search for minimal parameter uncertainties. In the low-heat limit, this algorithm nearly always converges on minimal parameter error for just three injections, the minimum needed to define the three fit parameters. This initially surprising result is a consequence of the nature of the experiment, whereby a fixed amount of "signal" (total reaction heat) is subdivided by the several injections.[4,9] Three injections unfortunately gives no margin of safety through redundancy, so I have devised a five-injection algorithm that does have such safety (two degrees of statistical freedom) while typically increasing the parameter uncertainties by only ~30%. For cases in which n is well-known in advance, it is permissible to fix it in the LS fit, giving even better determination of K and $\Delta H°$.[2,7] With n frozen, the optimization algorithm almost always converges on just two injections, so I have devised a four-injection prescription that provides a margin of safety. Finally, these results are tested on the complexation reaction of Ba^{2+} with 18-crown-6 ether,[10,11] illustrating some practical considerations for variable-v ITC experiments.

The variable-v programs devised here are based on the estimated experimental error properties of a specific instrument, MicroCal's VP-ITC;[6] however, an important result of the work in ref 6 was the confirmation that the experimental error becomes constant at low q, justifying the use of unweighted LS to analyze the data in such cases. Constant error should dominate for any ITC instrument at low q, so the variable-v schemes will be applicable to all, though the magnitudes of the parameter uncertainties will vary.

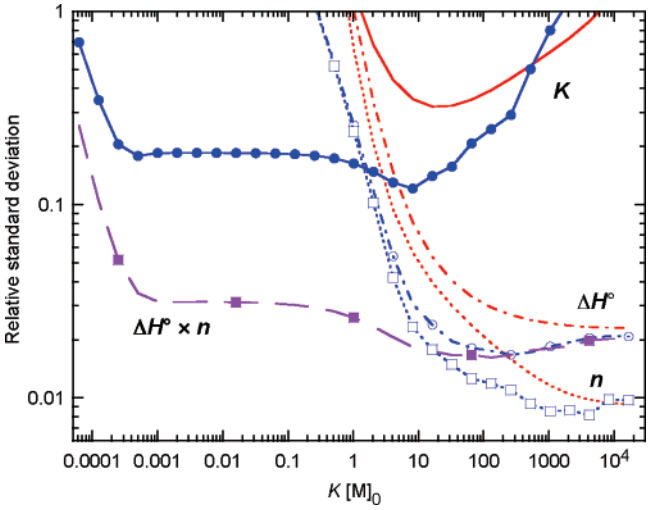

Figure 2. Relative standard deviation of fit parameters as a function of $c = K[M]_0$, for $h \equiv \Delta H°[M]_0 = 0.1$ cal/L. Curves with points included were computed for the 10-point procedure, with titration range R_m given by eq 1. Others were computed for titration to $R_m = 2$ using 25 injections. (The fitted product $\Delta H° \times n$, designated henceforth as H_n, is shown only for the 10-point procedure.)

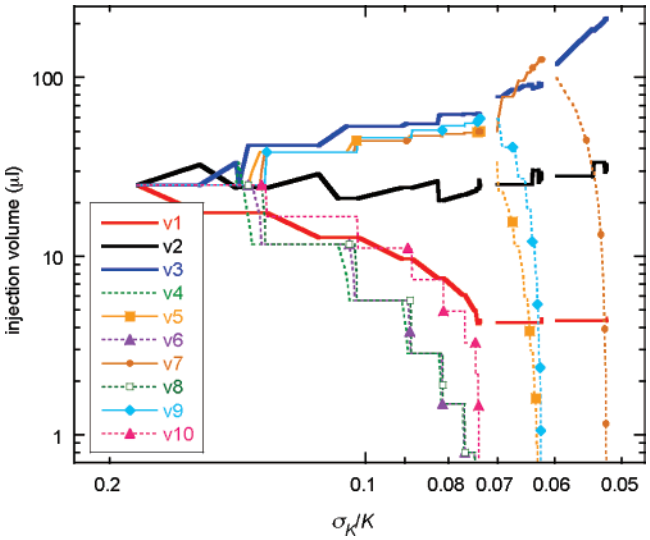

Figure 3. Performance of volume optimization algorithm for $h = 0.1$ cal/L and $c = 0.01$. The computation is started with 10 25-μL injections, is restarted with 6 injections near $\sigma_K/K = 0.07$, and is started once more with $m = 4$ near 0.06.

Materials and Methods

Computational Methods. As in ref 2, the approach of this study is the use of statistical data analysis to design experiments. From knowledge of how the data uncertainty depends on experimental parameters, we can predict the uncertainties in K, $\Delta H°$ (or H_n), and n from the least-squares a priori covariance matrix, $\mathbf{V}_{prior}$. Then by varying the experimental parameters, we identify combinations that minimize the uncertainties. The work in ref 2 covered the dependence on number of injections and stoichiometry range for injections of constant volume; here, I investigate variable injection volume.

The fact that the ITC fitting problem is a nonlinear one does not invalidate this approach. A Monte Carlo study of a number

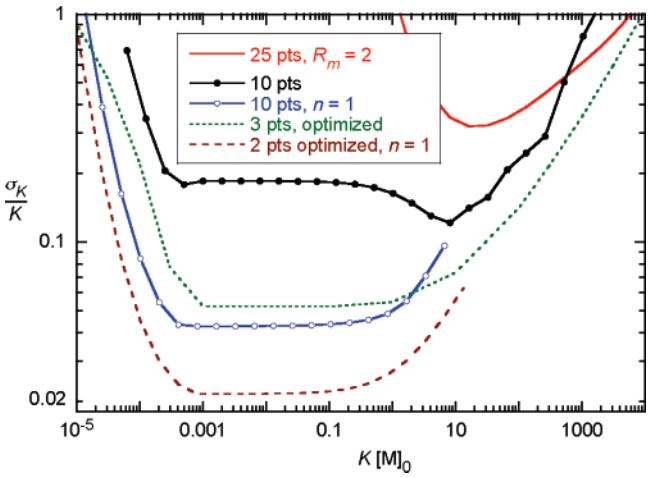

Figure 4. Relative standard deviation of K as a function of c. The two solid curves at top are repeated from Figure 2. The others represent the titrant-volume-optimized results for $m = 3$ (fitting all three parameters) and $m = 2$ (n frozen), and the results for the 10-injection algorithm with n frozen.

of common nonlinear fitting problems showed that the predicted uncertainties were trustworthy when the RSE was <0.1.[12] MC computations on the ITC fit model confirmed the validity of $\mathbf{V}_{\text{prior}}$ for the range $c = 1-1000$;[4] similar computations for a smaller c in ref 2 helped to identify the source of fitting problems in this regime, showing that $\Delta H°$ was strongly nonnormal, but that K, n, and H_n were well-behaved. Where RSEs are displayed below to >1, they cannot be trusted quantitatively, but there, the experiment fails to determine the parameters anyway.

In the low-h regime of emphasis here, the data uncertainty is practically constant (independent of q) for each injection. This constancy determines the derived results for optimal injection volumes, and only the scale of the resulting parameter uncertainties depends on the actual magnitude of the data uncertainty. The scale of the displayed RSEs is based on $\sigma_q = 0.8$ μcal from a generalized least-squares analysis of a large body of data for the Ba^{2+}−crown ether complexation reaction recorded on a MicroCal VP-ITC instrument.[6] For any other σ_q, the errors can be predicted by simple scaling: larger by 50%, for example, if $\sigma_q = 1.2$ μcal.

In perfusion-type ITC instruments, such as the VP-ITC, a volume v_i of the material in the cell is expelled from the active volume V_0 into the inactive fill region during the ith injection. Since injections of long time duration may be required in a variable-v program, the mixing process and the composition of the expelled material are of concern. In recent work, I considered two limiting models of the injection process, equivalent to instantaneous injection and instantaneous mixing.[13] The analysis of the heat of dilution data for NaCl(aq) showed surprisingly little sensitivity to the choice between these two models, even though the data included some large injections that lasted ~100 s. Accordingly, for the purpose of the present optimization computations, I use the simpler perfusion model (1) from ref 13, which assumes that the expelled material is of the prior equilibrium composition (instantaneous injection). Then the total concentrations of titrant and titrate (reacted plus unreacted) in the cell following the ith injection are

$$[\mathrm{X}]_{0,i} = [\mathrm{X}]_{0,i-1}(1 - f_i) + [\mathrm{X}]_{\mathrm{S}} f_i \tag{2}$$

and

$$[\mathrm{M}]_{0,i} = [\mathrm{M}]_{0,i-1}(1 - f_i) \tag{3}$$

where $[\mathrm{X}]_{\mathrm{S}}$ is the titrant concentration in the syringe and $f_i = v_i/V_0$. The concentrations for all injections are generated starting with $[\mathrm{X}]_{0,0} = 0$ and $[\mathrm{M}]_{0,0}$, the prepared concentration of titrate. The concentrations of complex $[\mathrm{MX}]_i$ are then computed from the equilibrium relation,

$$\frac{[\mathrm{MX}]_i}{([\mathrm{X}]_{0,i} - [\mathrm{MX}]_i)([\mathrm{M}]_{0,i} - [\mathrm{MX}]_i)} = K \tag{4}$$

from which the number of moles of complex produced by the ith injection is

$$\Delta n_i = V_0[\mathrm{MX}]_i - (V_0 - v_i)[\mathrm{MX}]_{i-1} \tag{5}$$

The heat q_i produced by the ith injection is thus $\Delta H°\Delta n_i$.

In early attempts to probe the dependence of the parameter uncertainties on injection volumes, I used a random number generator to break up the total titrant volume (typically 0.1−0.3 mL for $V_0 = 1.4$ mL) into m injections of varying v_i and then searched for v_i distributions that produced the lowest parameter standard errors. This approach succeeded in predicting the minimal uncertainties, but the resulting v_i distributions showed no clear patterns. Accordingly, I turned to an optimization scheme in which the volumes of adjacent injections were sequentially increased and decreased in trial-and-error fashion. Now some of the volumes in large-m procedures converged to zero. Dropping those injections and restarting the computation typically led to further $v_i = 0$, until eventually, almost every such optimization for $h = 0.1$ cal/L ended with just three finite v_i (two with n frozen).

A titration scheme with just enough injections to determine the parameters has obvious practical drawbacks, so I sought ways to optimize the v_i distributions for $m > 3$ (and 2 for n frozen) while constraining all v_i to remain finite. This required parametrizing the v_i distribution, and for reasons discussed below, I settled on the exponential expression

$$v_i = C \exp(b \times i) \tag{6}$$

with the constant C adjusted to keep the sum of the v_i equal to the total titrant volume, v_{tot}.

All optimization results discussed below employed eq 1 to set the titration range R_m. When this was relaxed, the optimization algorithm preferred larger R_m, apparently in an attempt to capture more of the heat of reaction present in the initial titrate. Since eq 1 already produces ~93% complexation in the low-c limit, the possible gains from increasing R_m are nominal.

Although the optimization can be with respect to either K or $\Delta H°$, all results given below were obtained by minimizing the uncertainty in K. This is because K is usually the more uncertain parameter—if H_n is considered in place of $\Delta H°$ at low c—and the volume distributions that optimize K are close to those that optimize H_n.

Experiments. At 25 °C, the complexation of Ba^{2+} with 18-crown-6 ether has $K \sim 6 \times 10^3$ L/mol and $\Delta H° \sim 8$ kcal/mol.[10,11] This reaction has customarily been studied by ITC with $[\mathrm{M}]_{0,0} = 1-10$ mM, giving a c in the range of 6−60 and $h = 8-80$ cal/L. Here, I have used $[\mathrm{M}]_{0,0} \sim 0.02$ mM, giving $c \sim 0.1$ and $h \sim 0.16$ cal/L, with both $BaCl_2$ and crown ether taken as titrate. The h figure means that there is a total heat of reaction of ~200 μcal. Other experimental procedures were as described previously.[11]

The experiments were conducted using an optimized program of three injections summing to $v_{\text{tot}} = 230$ μL: $v_1 = 4.3$ μL, $v_2 = 29.7$ μL, and $v_3 = 196$ μL. Both blanks (including water

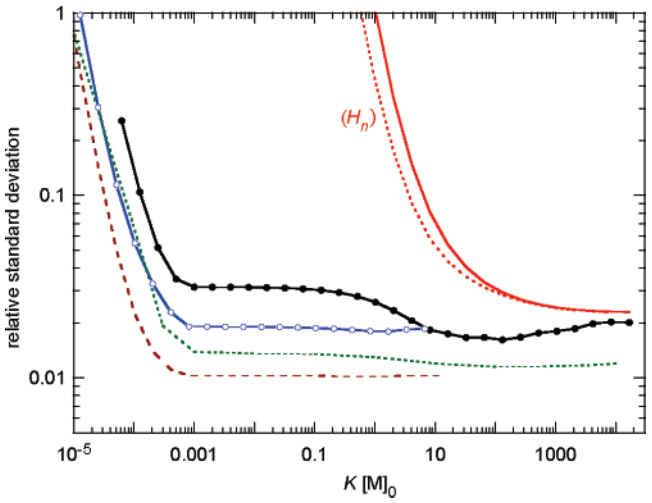

Figure 5. Relative standard deviation of $\Delta H°$ (n frozen) or H_n (n fitted) as a function of c. The top two solid curves are repeated from Figure 2; all are identified as in Figure 4, except the dashed curve labeled H_n, which is the counterpart to the solid curve for the 25-point scheme.

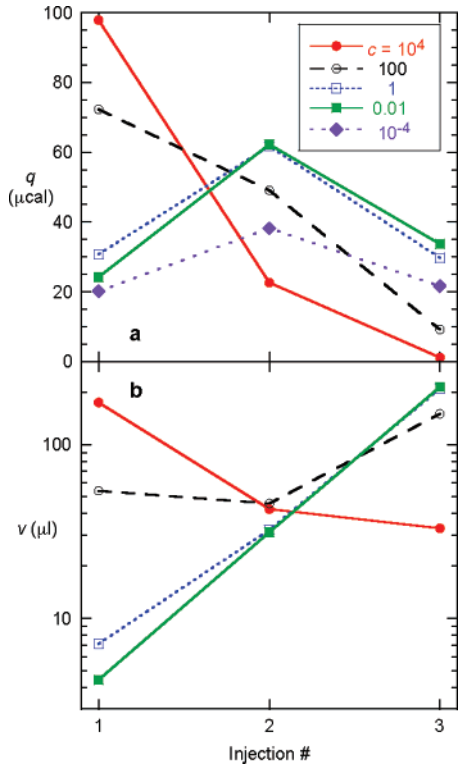

Figure 6. Dependence of q (a, top) and v (b) on injection number for optimized three-injection, three-parameter scheme, for $h = 0.1$ cal/L, $v_{tot} = 250$ μL, and selected values of c. Note logarithmic ordinate scale in part b.

into water) and the reactions were repeated a number of times to permit an evaluation of their statistics by sampling, for comparison with the predictions from the optimization calculations. Since no "throwaway" first injection was included, care was taken to avoid backlash[14] by preceding each experiment by a "down" motion of the syringe plunger before the syringe assembly was mounted on the cell. Because v_1 is small, backlash could otherwise be severe in this case.

Although the details of the mixing model are unimportant for the optimization computations, they are significant for the results in these experiments. To investigate this dependence, I have used the algorithm developed in ref 13 to bridge between the limits of instantaneous injection and instantaneous mixing. This is done by breaking up each injection into several increments, the first having volume v_{min} and subsequent having volume Δv, with each such increment treated by eqs 2−5 and the computed q's added to obtain the total q for that injection. Thus, for example, for a 200-μL injection, the choice $v_{min} = 50$ μL and $\Delta v = 1$ μL is equivalent to assuming that the material expelled in the first 50 μL of the injection has the preceding equilibrium composition, whereas after that, the composition is adjusted almost continuously. In the true situation, a long injection must surely permit some of the newly injected titrant to be expelled, but there must be concentration gradients present in the cell while the injection is in progress. By contrast, instantaneous mixing assumes no such concentration gradients, whereas instantaneous injection assumes they are so steep as to not permit newly injected material to reach the overflow region. The model of successive "equilibrium" injections does not model the concentration gradient problem, but it does seem to permit adequate accounting for it, as discussed below.

Results and Discussion

Injection Volume Optimization. For the computations described in this section, I have adopted 1 mol/L as the maximum allowed titrant concentration in the syringe $[X]_S$. This choice is somewhat arbitrary, but the problem is unavoidable at small c, because eq 1 yields $R_m = 13/c$ in this region, and $[X]_S \approx [X]_{0,m} V_0/v_{tot}$, giving $[X]_S \approx (13/c)(V_0/v_{tot})[M]_0$. To extend the region of high precision as far to low c as possible, I have also taken the total titrant volume, v_{tot}, to be as large as practicable for the VP-ITC (0.25 mL) and have assumed $\Delta H°$ = 10 kcal/mol and $[M]_{0,0} = 0.01$ mM. With decreasing c, the $[X]_S$ limitation eventually produces the upturns in the relative uncertainties in Figure 2 and similar figures below, because the reaction is progressively less complete at the end of the titration. This region is the only place where actual values of $[M]_{0,0}$ and $\Delta H°$ play a role; elsewhere, the curves are entirely determined by the choice $h = 0.1$ cal/L and the abscissum value of c. The curves for other h values shift up and down in inverse proportion to h for moderate changes in h. For example, the flat portion of the σ_K/K curve for the 10-injection scheme in Figure 2 drops below 10% when h is increased to 0.2 cal/L.

Figure 3 illustrates the injection volume optimization algorithm, starting with $m = 10$. The standard protocol gives $\sigma_K/K = 0.184$ for 10 25-μL injections. In the first cycle, four v_i's—nos. 4, 6, 8, and 10—go to zero. When the algorithm is restarted with $m = 6$, v_5 and v_9 vanish. In the restart with $m = 4$, v_7 drops out, leaving just the first three volumes finite and yielding $\sigma_K/K = 0.052$. The convergence on just three finite v_i was recognized as a consequence of the dominance of constant data error at low h, and most subsequent optimizations were done starting with $m = 4$ and equal v_i. When different starting v_i distributions were checked, they always converged on the same final distribution. (Some optimizations for large h, not considered here, did converge on >3 finite volumes; that behavior is due to the role of proportional data error at large h.[6])

In Figure 4, the results for K from Figure 2 are reproduced for comparison with three sets of results that significantly outperform the 10-injection scheme. Freezing n gives a 5-fold precision improvement, and another factor of 2 when the number of injections is reduced to two and their volumes optimized. These curves are shown only for low c, since it is normally better to fit n than freeze it at high c, even when it is thought to be well-known. The optimized 3-injection, 3-parameter scheme is a factor of 4 more precise than the 10-injection program in the low-c region and remains better than the other schemes at large c.

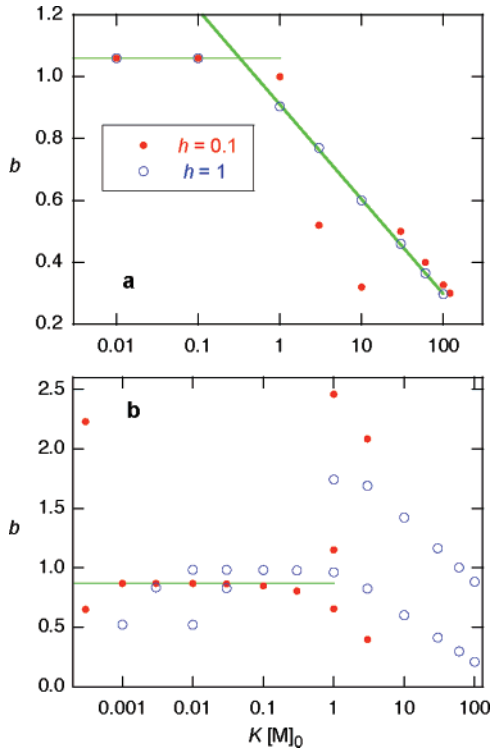

Figure 7. Optimization of exponential parameter b in representation of titrant volume v_i for five injections fitting three parameters (a) and four injections fitting two parameters (b). The horizontal straight lines are at $b = 1.06$ (a) and 0.87 (b); the sloping line in a is $b = 0.91 - 0.133 \ln(c)$. Note the multiple "local optima" in b (where more effort was directed at finding multiple values).

Figure 5 displays the relative standard deviation for $\Delta H°$ or H_n for the same conditions that yielded the results in Figure 4 for K. In all cases except the 25-injection scheme, the relative uncertainty is less than 3% down to $c < 0.001$. Thus, even though K is the optimization target, the results for $\Delta H°$ or H_n are more precise by factors of 2−6.

Figure 6 shows the heats and volumes from the optimized 3-injection scheme for selected c. At low c, the heats are approximately 1:2:1 in magnitude (except for $c = 10^{-4}$, where the limitation on $[X]_S$ is in effect). At the same time, the volumes become roughly exponentially distributed. This observation led to the use of eq 6 as a means to achieve partial optimization with more than the minimal number of injections. The subsequent optimization on K with respect to the exponential parameter b sometimes gave multiple results of comparable precision (within 10%). However, a significant number of these fell into simple patterns that led me to adopt the value $b = 0.87$ for the four-injection, two-parameter scheme (which is needed only for very small c), and the two-part definition for the five-injection, three-parameter scheme, as illustrated in Figure 7. Results for these two schemes are compared with their fully optimized progenitors in Figure 8, showing that the precision penalty for adding the two degrees of statistical freedom is <30% over much of the c regions of interest.

Figure 7 includes results for both $h = 0.1$ and 1.0 cal/L, and the latter were, in fact, used to define the sloping line in part a. As already noted, the multiple results for some points differ much less in actual efficiency than might be guessed from the range of optimized b values. The derived expressions for the dependence of b on c remain close to optimal for $h = 1$ cal/L, for which v_i optimization is not needed for precision but still might be useful for increased throughput.

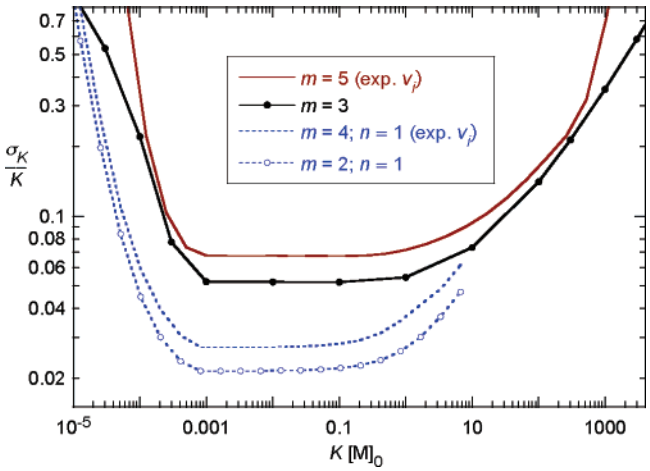

Figure 8. Comparison of relative uncertainties in K for exponential volume distributions (eq 6 and Figure 7) with those for fully optimized volume distributions (curves with points), for $h = 0.1$ cal/L. Solid curves show results for fitting all three parameters; dashed are for fitting two, with stoichiometry parameter fixed.

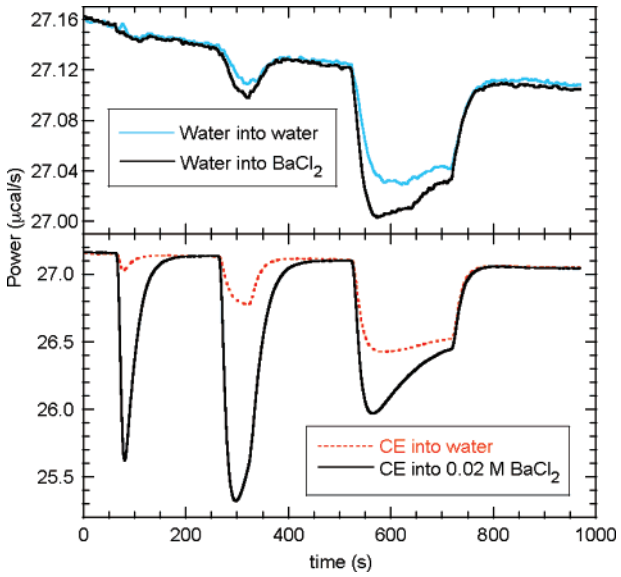

Figure 9. Blank and sample thermograms obtained for titration of 0.0212 mm $BaCl_2$ in water with 20.5 mM 18-crown-6 ether (CE) using optimized three-injection program. Titrant injection volumes were 4.3, 29.7, and 196 μL.

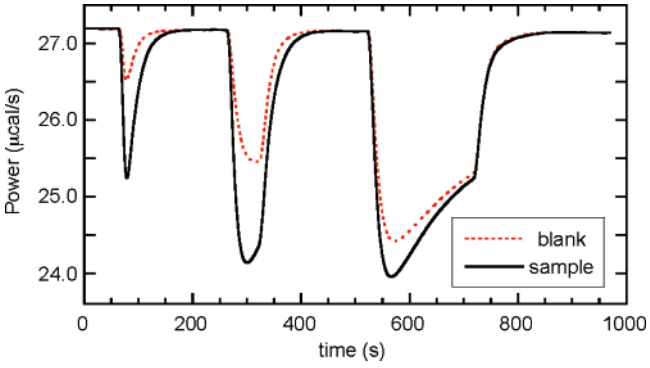

Figure 10. Blank and sample thermograms for titration of 0.0205 mM crown ether with 17.7 mM $BaCl_2$. Same titrant injection program as in Figure 9.

Experiments. Figures 9 and 10 show representative thermograms obtained in the experiments, and they illustrate immediately one complication of working in the low-c, low-h, low-

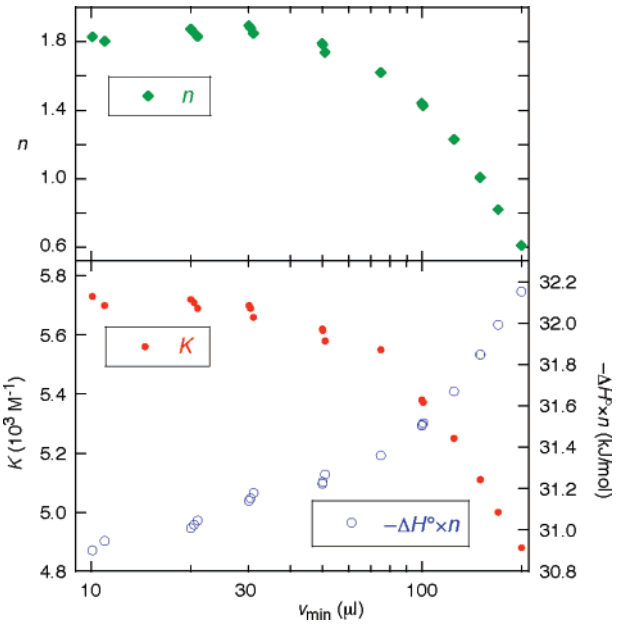

Figure 11. Average results for n (top), K, and $-\Delta H^\circ \times n$ from 30 combinations of 6 samples and 5 blanks obtained in experiments employing $BaCl_2$ as titrate (M), displayed as a function of the value of v_{min} used in the analysis. Multiple points close together employed different Δv values in the analysis; their abscissa values were taken as $v_{min} + \Delta v/v_{min}$. Note logarithmic scale in the v_{min} axis.

$[M]_0$ regime: The titrant concentrations $[X]_S$ are comparable to those required in more traditional ITC work, so the blanks may display q's comparable to those for the reaction itself. Then the reaction q's represent small differences between the sample and blank thermograms. In the case of M = $BaCl_2$, there is even a small heat signature for the dilution of the titrate (Figure 9, top), amounting to ~5 μcal for the last injection, which is more than 10% of the corresponding reaction q. The standard approach is to integrate the thermograms to obtain the estimated q's, then subtract the blank from the sample for each injection. While this procedure does not fully account for all aspects of the dilution heat,[11] it does properly cover them in the infinite dilution limit. Thus, that approach is used here, with additional subtraction of the net q's from the top thermograms in Figure 9 for the M = $BaCl_2$ runs.

For statistical sampling, each blank and each sample were run multiple times, and the blanks and samples were analyzed in all combinations. Figure 11 illustrates the average results for 30 such combinations in experiments employing $BaCl_2$ as titrate (Figure 9), displayed as a function of v_{min} in the analysis. These results also assumed that the first injection had a preinjection mixing "loss" of 0.1 μL.[14] This correction gave ~1% increase in K and ~1% decrease in H_n. On the other hand, neglecting the dilution heat for $BaCl_2$ (top of Figure 9) reduced K by 15% and increased H_n by 4%.

The results in Figure 11 show a strong dependence on v_{min} but are not very sensitive to Δv for subsequent increments of the injection, provided $\Delta v \leq v_{min}$. In part, this is because for the third injection, which is the only one affected for most v_{min} (since $v_2 < 30$ μL), most of the heat is captured in the first computational increment.

The stoichiometry number n is the most sensitive to v_{min} in the analysis, since this is also the most uncertain quantity. It is even possible for n to take on unphysical negative values (as it did in the analysis of the M = CE experiments for large v_{min}). However, the product H_n is well-determined and varies by only ~2% with changes in the fit model. Here, $n = 1$, so ΔH° is similarly well-determined.

TABLE 1: Results for Complexation of Ba^{2+} by 18-crown-6 Ether in Aqueous Reactions of $BaCl_2$ at 25 °C[a]

K (10^3 L/mol)	$-\Delta H^\circ$ (kJ/mol)	reference
5.9 (2)	31.42 (20)	10
5.14 (4)	31.42	16
5.1 (5)	33.2 (3)	17
5.40 (3)[b]	32.40 (11)[b]	6, 11
5.38 (21) [27][c]	31.5 (4) [4][c]	this work,[d] M = $BaCl_2$
5.21 (11) [14]	31.7 (3) [3]	this work, M = $BaCl_2$, $n = 1$
4.5 (3) [4]	33.0 (6) [7]	this work, M = CE
4.81 (11) [19]	32.5 (3) [5]	this work, M = CE, $n = 1$

[a] Figures in parentheses are standard errors, in terms of final displayed digits. [b] Values from ref 11, reanalyzed in ref 6, and corrected using calibration results from ref 13. Published values in ref 11 were $K = 5.56(4) \times 10^3$ L/mol and $\Delta H^\circ = 32.04(11)$ kJ/mol. [c] Figures in parentheses based on sampling of all combinations of blanks and samples; values in square brackets are predicted standard errors obtained as described in text. [d] All presented results from analysis using $v_{min} = 100$ μL; result for $-\Delta H^\circ$ is that for $-\Delta H^\circ \times n$ when n is fitted.

The results for K for $v_{min} \approx 100$ μL agree with our previous results (Table 1), although $-\Delta H^\circ$ is ~3% low at this point and remains ~1% low at maximum v_{min}. The results for titration of CE with $BaCl_2$ similarly span an ~15% range of K values and 4% range of H_n; however, the K values are ~15% smaller than those in Figure 11, and the $-H_n$ values are ~4% larger. (n values range from 0.5 to −1.) The reasons for these differences are not yet known, although it is possible that they relate to larger dilution heat effects when titrating with the relatively concentrated (18 mm) $BaCl_2$. The dilution heat anomalies vanish in the standard procedure of subtracting the blank from the sample if $BaCl_2$ and $Ba(CE)Cl_2$ have identical concentration dependences of their relative apparent molar enthalpy functions.[15] This holds for all similarly charged electrolytes in sufficiently dilute solutions, so it is a better approximation when $BaCl_2$ is used as titrate, in which case its concentration is 1000 times lower than when it is taken as titrant. Thus, at present, I have more confidence in those results, which also agree better with results from previous studies conducted at 100-fold larger c. The proper treatment of dilution heat effects in the more concentrated solutions is under continuing investigation.

When the data are reanalyzed with $n = 1$ (frozen), the results still depend on v_{min}, but less strongly by a factor of 2. For the data behind Figure 11, K ranges from 5.4 to 5.0, and $-H_n$ = 31.2−32.0 kJ/mol. For M = CE, there is a similar compression of the ranges, with K = 4.7−5.0 and $-H_n$ = 32.0−32.8 kJ/mol. The latter K estimates are higher than before, but still below other estimates.

The sampling statistics for the present experiments yield parameter standard errors somewhat smaller than predicted (Table 1). This may be due in part to too-conservative estimates of the data error at small q. However, it is also true that the ensemble statistics are low-biased: For a sum or difference of x and y, with statistics taken over all combinations of m values of x and n values of y, the apparent sampling variance, s^2, for $\nu = nm - 1$ is given by $\nu s^2 = n(m-1)s_x^2 + m(n-1)s_y^2$. For $s_x = s_y$ and m and n values of 5 and 6, the undershoot is 8%. The present situation is more complex, involving subtraction of the q's for three points in each experiment. Still, this effect is likely <10%.

Conclusion

ITC experiments can be run successfully at very small values of c, but this region presents special problems. First, to obtain

precise estimates of K and $\Delta H°$, one must titrate to large stoichiometric excess of titrant over titrate. Then the reaction heat is strongly distributed toward the early part of the titration. In addition, n and $\Delta H°$ become highly correlated so that neither can be determined individually. However, their product is statistically well-defined; and when n is known from other work, it can be frozen in the least-squares analysis, leading to even better precision in the estimation of both $\Delta H°$ and K.

By varying the injection volume of titrant, one can achieve significant improvement over results obtained using a previously recommended 10-injection protocol. In the limit of low total reaction heat (as can be encountered for low $[M]_0$, low $\Delta H°$, or both), best precision comes with just the minimal number of injections: three if n is fitted, two if not. Since these choices give no margin of safety through statistical degrees of freedom, I have devised five- and four-injection procedures with variable injection volumes, v_i, dictated by an exponential formula. These procedures give an ~30% rise in the parameter standard deviations over best results.

Tests of these results on the familiar complexation reaction of Ba^{2+} with 18-crown-6 ether at $c = 0.1$ and $h = 0.16$ cal/L illustrate some practical considerations and confirm that reliable results can be obtained from ITC in this regime: K within 5% when n is fitted, 2% when it is frozen, and $\Delta H°$ (or $\Delta H° \times n$) values within ~2%. There is a significant undershoot in the K estimates when $BaCl_2$ is used as titrant, which is tentatively attributed to heat of dilution anomalies for the relatively large $BaCl_2$ concentrations needed in those experiments.

The a priori approach to the estimation of the LS fit parameter errors in this work assumes prior knowledge of the data error. Those who analyze their data with standard software usually do unweighted fitting and estimate the parameter uncertainties in the a posteriori mode. That is fine for analysis of low-h data, where constant uncertainty in q dominates. For the four- and five-injection methods devised here, the only drawback is that the resulting parameter standard deviations are themselves highly uncertain, since the RSE in an estimated σ is $1/\sqrt{2\nu}$, or 50% for $\nu = 2$.

The observation of best agreement with prior results for $v_{min} \approx 100\ \mu$L is surprising, since it implies that negligible loss of titrant to the cell overflow region occurs in the first ~100 s for the default stirring rate of 300/s. Similar results were found in the calibration study,[13] which also revealed a need to wait a long time for final equilibration after large injections. The matter of the proper fitting model will require further study, but the model dependence anyway amounts to uncertainties of only ~2% in K and ~1% in $\Delta H°$ in the present case, when v_{min} is set at 100 μL and n is frozen. The latter choice is the only mode for useful ITC at very low c. The fit model dependence should not change much in this region and will be unimportant in practically all applications. In addition, this dependence is a direct consequence of the large total titrant volume,[13] so it can be made negligible by using $v_{tot} < 100\ \mu$L if the required higher syringe concentrations can be tolerated.

Although the emphasis in the present work has been on getting ITC results under extreme conditions of low c and low h, the devised small-m injection schemes can also be used to enhance throughput in situations in which volume optimization is otherwise not needed. The three-injection experiments described here had run times of ~15 min but could have been shorter without the intentional inclusion of a 200-μL third injection. For more routine situations not requiring large injection volumes, five to six injections can be completed in 15−20 min. This is comparable to the time needed for the single-injection scheme,[18] which is praised for its throughput advantage. Additionally, the standard method should be less subject to problems stemming from the remaining uncertainties about the mixing processes in the cell.

At high c, the v_i optimization results are sensitive to exactly how the heat is sampled, particularly with a small number of injections. Still, the simple prescription derived in Figure 7a works well up to $c = 500$. Determination of K beyond $c = 10^4$ requires a larger h for any titration scheme. Alternatively, many binding processes with very large K can be studied by competitive binding,[19] which in effect shifts the titration curve down to smaller c. There, the methods discussed here can again be used to optimize performance.

Final cautionary reminders: Both the 10-injection protocol recommended in ref 2 and the volume optimizations developed here are premised upon confident knowledge that the process under study is 1:1 binding. The 10-injection protocol has enough degrees of freedom to identify breakdown of this assumption under some circumstances. But none of these methods will determine n and $\Delta H°$ independently below $c = 1$ for $h = 0.1$ cal/L, nor below $c \approx 0.1$ even for $h = 10$ cal/L.[2] Thus, successful use of ITC for $c < 0.1$ really requires independent knowledge of n, as, in fact, do most other methods for studying binding.[8] The devised four- and five-injection schemes provide a way to push ITC in the low-h limit, where such optimization can make the difference between failed and successful experiments. The exponential titrant volume prescriptions remain reliable up to $h = 1$ cal/L, where such optimization is normally not needed but might still offer time savings and enhanced throughput in repetitive work. However, for $h = 1$ cal/L, even the 10-injection protocol gives 2% error in K, and at that level, systematic errors will often render volume optimization meaningless. The v_i optimization procedures should not be used for h much higher than 1 cal/L, because there, the small numbers of injections can give q's that exceed the high-signal capacity of the instrument, invalidating those data. From such practical concerns, even large-m experiments run at very small c and high h may require the use of small, early injections.

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