Appendix: Equations Used for Fitting ITC Data

I. General Considerations

It will be assumed throughout that the macromolecule M is in the cell at an initial bulk concentration \( M_t^o \) (moles/liter) before the first injection, and the ligand X to be injected is initially at zero concentration in the cell. The working volume (cross-hatched area below) of the lollipop-shaped cell is \( V_o \), the size of the \( i \)th injection is \( \Delta V_i \) and the total liquid which has been injected at any point during the experiment, \( \Delta V \), is simply the sum of the individual \( \Delta V_i \) for all injections.

At the beginning of an experiment, both the cell and the long communication tube are filled with macromolecule solution, but it is only that contained within \( V_o \) that is sensed calorimetrically. Because of the total-fill nature of the cell each injection acts to drive liquid out of the working volume and up into the inactive tube as shown by the darkened portion representing \( \Delta V \). Thus, the concentration of macromolecule in \( V \) changes a small amount with each injection since the total number of moles of macromolecule initially in \( V \) (i.e. \( M_t^o \) times \( V_o \)) at the beginning of the experiment is later distributed in a larger volume, \( V_o + \Delta V \). Since the average bulk concentration of macromolecule in \( \Delta V \) is the mean of the beginning concentration \( M_t^o \) and the present concentration \( M_t \) in the active volume, then conservation of mass requires that

\[
M_t^o V_o = M_t V_o + \frac{1}{2} \left( M_t + M_t^o \right) \Delta V \quad (1)
\]

so that

\[
M_t = M_t^o \left( \frac{1 - \frac{\Delta V}{2V_o}}{1 + \frac{\Delta V}{2V_o}} \right) \quad (2)
\]

Using similar reasoning, it is easily shown that the actual bulk concentration of ligand in \( V_o \), \( X_t^o \), is related to the hypothetical bulk concentration \( X_t^o \) (assuming that all of the injected ligand remained in \( V_o \)) as follows:

\[
X_t^o V_o = X_t V_o + \frac{1}{2} X_t \Delta V \quad (3)
\]
\[ X_t = X_t^o \left( \frac{1}{1 + \frac{\Delta V}{2V_o}} \right) \]  

(4)

The above expressions for \( M_t \) and \( X_t \) are used by Origin to correct for displaced volume effects which occur with each injection.

**II. Single Set of Identical Sites**

In the following equations,

- \( K = \) Binding constant;
- \( n = \# \) of sites;
- \( V_o = \) active cell volume;
- \( M_t \) and \([M]\) are bulk and free concentration of macromolecule in \( V_o \);
- \( X_t \) and \([X]\) are bulk and free concentration of ligand, and
- \( \Theta = \) fraction of sites occupied by ligand \( X \).

\[ K = \frac{\Theta}{(1 - \Theta)[X]} \]  

(5)

\[ X_t = [X] + n\Theta M_t \]  

(6)

Combining equations (5) and (6) above gives

\[ \Theta^2 - \Theta \left[ 1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} \right] + \frac{X_t}{nM_t} = 0 \]  

(7)

The total heat content \( Q \) of the solution contained in \( V_o \) (determined relative to zero for the unliganded species) at fractional saturation \( \Theta \) is

\[ Q = n\Theta M_t \Delta H V_o \]  

(8)

where \( \Delta H \) is the molar heat of ligand binding. Solving the quadratic equation (7) for \( \Theta \) and then substituting this into eq. (8) gives

\[ Q = \frac{nM_t \Delta H V_o}{2} \left[ 1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}} \right] \]  

(9)

The value of \( Q \) above can be calculated (for any designated values of \( n, K, \) and \( \Delta H \)) at the end of the \( i^{th} \) injection and designated \( Q(i) \). The parameter of interest for comparison with experiment, however, is the change in heat content from the completion of the \( i-1 \) injection to completion of the \( i \) injection. The expression for \( Q \) in eq. (9) only applies to the liquid contained in volume \( V_o \). Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e., \( \Delta V_i = \) injection volume) since some of the liquid in \( V_o \) after the \( i-1 \) injection will no longer be in \( V_o \) after the \( i^{th} \) injection, even though it will contribute to the heat effect (assuming the kinetics of reaction and mixing are fast) before it passes out of the working volume \( V_o \). The liquid in the displaced volume contributes about 50%
as much heat effect as an equivalent volume remaining in \( V_o \). The correct expression then for heat released, \( \Delta Q(i) \), from the \( i^{th} \) injection is

\[
\Delta Q(i) = Q(i) + \frac{dV_i}{V_o} \left[ \frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1) \tag{10}
\]

The process of fitting experimental data then involves 1) initial guesses (which most often can be made accurately enough by Origin) of \( n, K, \) and \( \Delta H \); 2) calculation of \( \Delta Q(i) \) for each injection and comparison of these values with the measured heat for the corresponding experimental injection; 3) improvement in the initial values of \( n, K, \) and \( \Delta H \) by standard Marquardt methods; and 4) iteration of the above procedure until no further significant improvement in fit occurs with continued iteration.

III. Two Sets of Independent Sites

Using the same definition of symbols as above for set 1 and set 2, we have

\[
K_1 = \frac{\Theta_1}{(1-\Theta_1)X} \quad K_2 = \frac{\Theta_2}{(1-\Theta_2)X} \tag{11}
\]

\[
X_i = [X] + M_i(n_1\Theta_1 + n_2\Theta_2) \tag{12}
\]

Solving equation (11) for \( \Theta_1 \) and \( \Theta_2 \) and then substituting into equation (12) gives

\[
X_i = [X] + \frac{n_1M_i[X]K_1}{1+[X]K_1} + \frac{n_2M_i[X]K_2}{1+[X]K_2} \tag{13}
\]

Clearing equation (13) of fractions and collecting like terms leads to a cubic equation of the form

\[
[X]^3 + p[X]^2 + q[X] + r = 0 \tag{14}
\]

where

\[
p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2)M_i - X_i
\]

\[
q = \left( \frac{n_1}{K_2} + \frac{n_2}{K_1} \right)M_i - \left( \frac{1}{K_1} + \frac{1}{K_2} \right)X_i + \frac{1}{K_1K_2}
\]

\[
r = \frac{-X_i}{K_1K_2} \tag{15}
\]

\(^1\) The first infinitesimal volume element in the \( i \) injection contributes no heat effect since it has already equilibrated at existing concentrations after the \( i-1 \) injection. The last volume element of an injection contributes heat effects equal to the liquid remaining in \( V_o \) since its concentrations are equivalent to those in \( V_o \) after the \( i \) injection. Assuming linearity over the small \( \Delta V_i \) volume increment, then the liquid in the displaced volume is only half as effective in producing heat relative to the liquid in \( V_o \).
Appendix: Equations Used for Fitting ITC Data

Equations 14 and 15 can be solved for \([X]\) either in closed form or (as done in Origin) numerically by using Newton’s Method if parameters \(n_1, n_2, K_1,\) and \(K_2\) are assigned. Both \(\Theta_1\) and \(\Theta_2\) may then be obtained from equation 11 above.

As discussed earlier in section II, the heat content after any injection \(i\) is equal to

\[
Q = M_i V_i \left( n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2 \right)
\]  
(16)

After a similar correction for displaced volume, the pertinent calculated heat effect for the \(i\) injection is

\[
\Delta Q(i) = Q(i) + \frac{dV_i}{V_o} \left[ \frac{Q(i) + Q(i - 1)}{2} \right] - Q(i - 1)
\]  
(17)

which may be used in the Marquardt algorithm to obtain best values for the six fitting parameters.

IV. Sequential Binding Sites

For sequential binding, the binding constants \(K_1, K_2, \ldots, K_n\) must be defined relative to the progress of saturation, so that

\[
K_1 = \frac{[MX]}{[M][X]} \quad K_2 = \frac{[MX_2]}{[MX][X]} \quad K_3 = \frac{[MX_3]}{[MX_2][X]}
\]  
(18)

In the sequential model, there is no distinction as to which sites are saturated, but only as to the total number of sites that are saturated. If the sites are identical, then there is a statistical degeneracy associated with the sequential saturation since the first ligand to bind has more empty sites of the same kind to choose from than does the second ligand, etc. For identical interacting sites then, we can distinguish between the phenomenological binding constants \(K_i\) (defined by eq (18)) and the intrinsic binding constants \(K_i^o\) where the effect of degeneracies has been removed. The relationship between the two binding constants is given by:

\[
K_i = \frac{n - i + 1}{i} K_i^o
\]  
(19)

All calculations given below, as well as parameters reported from curve-fitting, are in terms of \(K_i\) values but the operator may convert to \(K_i^o\) values, if desired, using eq (19). Since concentrations of all liganded species \([ML_i]\) can be easily expressed in terms of the concentration of the non-liganded species, \([M]\), then the fraction of total macromolecule having \(i\) bound ligands, \(F_i\), is simply
\[
F_o = \frac{1}{P}
\]

\[
F_i = \frac{K_i[X]}{P}
\]  \hspace{1cm} (20)

\[
F_2 = \frac{K_iK_2[X]^2}{P}
\]

\[
F_n = \frac{K_iK_2\ldots K_n[X]^n}{P}
\]

where

\[
P = 1 + K_1[X] + K_1K_2[X]^2 + \ldots + K_1K_2\ldots K_n[X]^n
\]  \hspace{1cm} (21)

\[
X_t = [X] + M_t \sum_{i=1}^{n} iF_i
\]

Once \( n \) and values of fitting parameters \( K_1 \) through \( K_n \) are assigned, then equations (20) - (21) may be solved for \([X]\) by numerical methods (the Bisection method is used). After \([X]\) is known, all \( F_i \) may be calculated from equation (20) and the heat content after the \( i \)th injection is determined from

\[
Q = M_iV_o \left( F_1\Delta H_1 + F_2\left[ \Delta H_1 + \Delta H_2 \right] + \ldots + F_n\left[ \Delta H_1 + \Delta H_2 + \Delta H_3 + \ldots \right] \right)
\]  \hspace{1cm} (22)

And, as before,

\[
\Delta Q(i) = Q(i) + \frac{dV_i}{V_o} \left[ \frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1)
\]  \hspace{1cm} (23)

Which then leads into the Marquardt minimization routine.
Appendix: Equations Used for Fitting ITC Data

V. Enzyme/substrate/inhibitor Assay

Assaying enzymes, inhibitors or substrates by calorimetric activity has the major advantage that it works well for any enzyme/substrate/inhibitor system with no prior chemical modification of any participants in the reaction. The rate $R_t$ of the substrate decomposition reaction is directly proportional to the power output in the calorimeter cell, i.e.,

$$R_t = \frac{P}{\Delta H V_0}$$

where $P$ is the power generated by the reaction, $\Delta H$ is the heat of decomposition of the substrate, and $V_0$ is the cell volume. The units of $R_t$ will be moles/l/sec if $P$ is expressed in $\mu$cal/sec, $\Delta H$ in $\mu$cal per mole of substrate, and $V_0$ in liters, for example.

If Michaelis-Menten kinetics are assumed then the experimental values for the rate $R_t$ can be expressed as

$$R_t = \frac{k_{cat} [E]_{cat} [S]_r}{[S]_r + K_M \left(1 + \frac{[I]}{K_I}\right)}$$

where $k_{cat}$ is the catalytic rate constant for substrate decomposition, $K_M$ is the Michaelis constant, $[E]_{tot}$ is the total enzyme concentration, and $[S]_t$ is the instantaneous concentration of substrate. The equation as written is valid both in the absence or presence of a competitive inhibitor at concentration $[I]$ and with inhibition constant $K_I$.

The use of equation (25) assumes no effects from product inhibition. This assumption has been discussed by Todd and Gomez (Todd, M. J. & Gomez, J. (2001) Analytical Biochemistry 296, 179-187.) and found to be quantitative in many cases. In those cases where product inhibition is significant, then equation (25) can only be used to express initial rates of reaction prior to accumulation of product.

Todd and Gomez discussed in some detail the two methods by which assays can be carried out in a titration calorimeter, and these are summarized below.

Method 1: Single injection. Using this approach, the reaction is initiated by injecting enzyme solution from the syringe into the sample cell containing substrate solution, or vice versa. If desired, a competitive inhibitor may also be included in one solution or the other. The reaction is allowed to go to completion in the calorimeter cell, and the power $P$ is recorded as a function of time $t$.

Integration of the excess power $P$ associated with the reaction enables $\Delta H$ to be determined, i.e.,

$$\Delta H = \int_0^\infty P \, dt$$

Method 2: Initial rate. In this approach, the reaction is initiated by injecting enzyme solution from the syringe into the sample cell containing substrate solution. The reaction is allowed to go to completion in the calorimeter cell, and the power $P$ is recorded as a function of time $t$.

Integration of the excess power $P$ associated with the reaction enables $\Delta H$ to be determined, i.e.,

$$\Delta H = \int_0^\infty P \, dt$$
where \( [S]_{t=0} \) is the starting substrate concentration. Knowing \( \Delta H \), the substrate concentration can be determined as a function of time from the equation:

\[
[S]_t = [S]_{t=0} - \frac{\int_0^t Pdt}{\Delta H V^0}
\]  

(27)

After obtaining the time-dependent rate from equation (24), then these data can be equated to the Michaelis expression in equation (25) to provide the final equation to be fit by non-linear least squares. In the absence of inhibitor, \( k_{cat} \) and \( k_M \) are used as variable parameters during iterative fitting. In the presence of inhibitor \( I \), it is best to enter previously determined values of \( k_{cat} \) and \( k_M \) and use \( K_I \) as the only variable fitting parameter.

**Method 2: Multiple injections.** In this method, multiple injections of substrate solution from the syringe are made into the reaction cell containing enzyme solution (with or without inhibitor). After each injection, a sufficient time is allowed for the instrument to equilibrate at the new power level resulting from the increased substrate concentration. Measurements are carried out quickly enough however so that little hydrolysis of substrate takes place relative to the total substrate contained in the cell. That is, \( [S]_t \) is calculated directly from the total added substrate assuming no significant hydrolysis.

Equations (24)-(25) are still valid for Method 2, except that \( R_t \) and \( [S]_t \) now correspond to discrete values of the rate and substrate concentration after each injection, rather than time-dependent values. To determine \( \Delta H \) from equation (26), it is necessary to carry out another single-injection experiment where hydrolysis is allowed to go to completion. Having done this, then discrete values of \( R_t \) at different \( [S]_t \) are calculated, so that equation (25) can then be fit to obtain best values of \( k_{cat} \) and \( k_M \) (in the absence of inhibitor). In the presence of a competitive inhibitor, data are also fit to equation (25) but using \( k_{cat} \) and \( k_M \) as fixed (results obtained from previous experiment with no inhibitor present) and treating \( K_I \) as the only fitting parameter.

**VI. Dimer Dissociation Model**

A protein molecule \( P \) may associate at high concentrations to form a dimer. The dilution of this concentrated protein solution by injection into the calorimeter cell containing buffer can then result in some heat effects from dissociation

\[
P_2 \xleftrightarrow{\Delta H_{disc}} 2P
\]

\[
K = \frac{(P)^2}{(P_2)}
\]

where \( (P) \) and \( (P_2) \) are the concentrations of monomer and dimer and where \( \Delta H_{disc} \) is the heat of dissociation of the dimer. It is assumed in this model that the stoichiometry is well-defined, i.e., no aggregates with stoichiometry higher than 2 are present. By measuring heats for a series of injections it is then possible, using curve-fitting, to determine the dissociation constant \( K \), and heat of dissociation.
The equivalent monomer concentration after the ith injection, Ci, is the sum of the actual monomer concentration (P)i plus 2 times the aggregate concentration (P2)i. Using the expression for the dimer dissociation constant to obtain (P)i in terms of (P2)i, leads to the equation

\[ C(i) = K^{1/2} (P_2)_i^{1/2} + 2(P_2)_i \]  

(28)

A similar expression applies to the solution in the syringe of fixed concentration Csyr

\[ C_{syr} = K^{1/2} (P_2)_{syr}^{1/2} + 2(P_2)_{syr} \]  

(29)

Since Csyr is known and C_i can be determined from Csyr knowing injection volumes, then (P_2)_syr and (P_2)_i can be determined from equations (28)-(29) if K is assigned.

The heat released q_i when the ith injection of volume dV_i is made into a fixed-volume (V_o) cell will be

\[ q_i = \Delta H_{disc} (P_2)_{syr} dV_i - \Delta H_{disc} [(P_2)_i - (P_2)_{i-1}] \left[ V_0 + \frac{dV_i}{2} \right] \]  

(30)

The first term in equation (30) is the heat content of the aggregate contained in the injection volume prior to injection while the second term is the net heat content due to the difference in aggregate present in the cell before and after the injection. The \( V_0 + \frac{dV_i}{2} \) factor in the final term is an effective volume which takes into account the displacement which occurs in a total-fill cell (see Appendix, section I).

Assuming experimental parameters V_o, dV_i, and Csyr are known, equations (28)-(30) are simultaneous equations which can be solved for q_i whenever values are assigned to K and \( \Delta H_{disc} \). Only the latter two parameters are varied during iterative fitting.

VII. Competitive Binding Model

Using conventional ITC methods, binding constants from 10^3 M^-1 to 10^8 M^-1 can be measured most accurately. When binding constants significantly exceed 10^8 M^-1, instrument sensitivity becomes challenged as concentrations are lowered to the point where quantitative measurements of the binding constant would be possible. On the other hand, binding constants substantially in excess of 10^8 M^-1 can be measured quantitatively if such strong-binding ligands are studied in competition with a second ligand which binds competitively but more weakly to the same binding site.
Competitive binding studies are carried out using the strong-binding ligand A as the injectant, with the solution in the cell containing the second competitive ligand B as well as the binding protein P (or other target molecule). This system then has two equilibria which are displaced with each injection, i.e.,

\[
\begin{align*}
A + P \rightleftharpoons P A & \quad K_A = \frac{[PA]}{[P][A]} \\
B + P \rightleftharpoons P B & \quad K_B = \frac{[PB]}{[P][B]}
\end{align*}
\]

The value of \( K_B \) and \( \Delta H_B \) for the competing ligand are first measured in a conventional ITC experiment, and these parameter values are entered as known parameters when determining \( K_A \) from results of the competition experiment. For the competition experiment, the total concentration of competing ligand, \([B]_{\text{tot}}\), should be selected such that

\[
\frac{K_A}{K_B[B]_{\text{tot}}} \approx 10^{-5} - 10^8 \text{ M}^{-1}
\]

where "\( K_A \)" is the estimated value of \( K_A \).

The detailed equations used in the fitting model for competitive binding are found in a paper by Sigurskjold (Sigurskjold, B. W. (2000) *Analytical Biochemistry* 277, 260-266.). MicroCal would like to thank Dr. Sigurskjold for providing this fitting routine.

**VIII. Single Injection Method**

*Creating New Worksheet.* The raw data (after time constant correction, Fourier filtering, baseline subtraction, and eliminating inappropriate data) are used to form a new worksheet which is modeled after the existing worksheet used with multi-injection binding data.

1) **Input Parameters.** In addition to the raw data parameters (\( \Delta P \) (ucal/sec) from the Y axis and time \( t \) (sec) from the X axis of the corrected raw data, the known parameters are the injection rate \( R \) (\( \mu \)l/sec, stored in header), total delivery volume \( V_{\text{inj}} \) (ul, stored in header), active cell volume \( V_{\text{cell}} \) (ml), the initial macromole concentration in the cell \( M^* \) (mM) before any dilution, the dilution factor \( d_M \) for the macromolecule solution resulting from autosampler loading, the initial ligand concentration in the syringe \( X^* \) before any dilution, the dilution factor \( d_X \) for the ligand concentration resulting from loading. The approximate values are 0.95 for \( d_M \) and 0.91 for \( d_X \), and the values will be independent of the instrument which is used in the experiment.

2) **Point numbering.** In the existing Origin worksheet for multiple injections, the rows are numbered 1,2,3, \ldots according to the injection number. In the worksheet for single injection experiments, the numbering corresponds to the data point number. The data points will be spaced at one for each filter period (2 sec).
3) DH and time t columns. The DH column corresponds to the column of the same name in the existing Origin ITC worksheet while the time t column is one which doesn’t exist in the existing worksheet and must be added. The DH and time t columns should be filled with the data points from the above data set (after TC correction, FT smoothing, control subtraction, and data trimming). DH is the Y axis value \( \Delta P \) (ucal/sec) and time t (sec) is the corresponding X axis value.

4) INJV column. All entries into this column should be identical and equal to the injection rate \( R \) (ul/sec) times the filter time (2 sec).

5) \( X_t \) column. \( X_t = \left( \frac{X^0 d x}{1000 V_{cell}} \right) \left( 1 - \frac{R t}{2000 V_{cell}} \right) \)

6) \( M_t \) column. \( M_t = M^0 d_m \left( \frac{1 - \frac{r t}{2000 V_{cell}}}{1 + \frac{r t}{2000 V_{cell}}} \right) \)

7) \( XM_t \) column. \( XM_t = \frac{X_t}{M_t} \)

Note: Indexing for \( X_t, M_t, \) and \( INJV \) refer to values before the ith injection, while DH, XM, NDH refer to indexing after the ith injection (the new column time t is also indexed after the ith injection).