ITC XXIX – Instrumentation and data analysis

Beaudette N. V. and Langerman N. (1978) An improved method for obtaining thermal titration curves using micromolar quantities of protein. *Anal Biochem* **90,** 693-704.

Berger R. L., Cascio H. E., Davids N., Gibson C. G., Marini M., and Thiebault L. (1985) An automated differential thermal and potentiometric titration apparatus for binding studies. *J Biochem Biophys Methods* **10,** 245-259.
Abstract: A differential pH-thermal titration apparatus is described which can detect pH differences with a sensitivity of +/- 0.0001 pH units and a thermal sensitivity of +/- 0.00002 degree C at a time constant of 0.1 s. With a reaction which yields 1 kcal mol-1, the current system can detect concentrations as low as 4 X 10^{-6} M or, in a 2 ml volume, a total amount of 40 nmol. With a time constant of 0.1 s, the sensitivity is 20 +/- 4 µ degrees C. The experimental protocol is specified by a microprocessor and three modes of operation are possible: titration at constant rate of reagent addition, titration at variable rates of addition so that the contents of both cells are at either constant pH or at a constant temperature and variable rate when a rate of change is specified. Experimental data are collected in files, corrected for heat loss, initial baseline drift, and changes in volume. The final corrected data from the standardized run of 0.01338 M HCl in 0.2 M KCl at 25 degrees C calibrate the pH scale and yield the calorimetric conversion constants and pKw which are calculated and stored for subsequent corrections for the titration of an unknown acid or the measurement of binding constants and heats.

Berger R. L., Mudd C. P., Clem T., Kolobow T., Beile E., Simons P. C., Michel S., and McClintock W. (1989) A stopped-flow mixer device for a batch microcalorimeter application to NAD-NADase reaction. *J Biochem Biophys Methods* **18,** 113-124.
Abstract: A new molded polypropylene, diamond-like carbon (DLC)-coated mixing cell has been developed for use in the batch microcalorimeter. Reagent volume can be varied from 25 µliters to 100 µliters. A 10 µcalorie reaction heat can be measured to 5%. Repeat reactions can be done as often as every 10 min for a fast reaction. Reactions can be started within 1 h or less after loading. A pre-equilibrator and a temperature-controlled syringe drive unit permit solutions to be stored at 4 degrees C while being run at any temperature from -20 degrees C to 40 degrees C. The kinetics and enthalpy of reaction of NAD-NADase have been measured. Δ H is about 21 kcal/mol endothermic.

Cedervall T., Lynch I., Lindman S., Berggard T., Thulin E., Nilsson H., Dawson K. A. and Linse S. (2007) Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc. Natl. Acad. Sci U. S. A* **104,** 2050-2055.
Abstract: Due to their small size, nanoparticles have distinct properties compared with the bulk form of the same materials. These properties are rapidly revolutionizing many areas of medicine and technology. Despite the remarkable speed of development of nanoscience, relatively little is known about the interaction of nanoscale objects with living systems. In a biological fluid, proteins associate with nanoparticles, and the amount and presentation of the proteins on the surface of the particles leads to an in vivo response. Proteins compete for the nanoparticle "surface," leading to a protein "corona" that largely defines the biological identity of the particle. Thus, knowledge of rates, affinities, and stoichiometries of protein association with, and dissociation from, nanoparticles is important for understanding the nature of the particle surface seen by the functional machinery of cells. Here we develop approaches to study these parameters and apply them to plasma and simple model systems, albumin and fibrinogen. A series of copolymer nanoparticles are used with variation of size and composition (hydrophobicity). We show that isothermal titration calorimetry is suitable for studying the affinity and stoichiometry of protein binding to nanoparticles. We determine the rates of protein association and dissociation using surface plasmon resonance technology with nanoparticles that are thiol-linked to gold, and through size exclusion chromatography of protein-nanoparticle mixtures. This method is less perturbing than centrifugation, and is developed into a systematic methodology to isolate nanoparticle-associated proteins. The kinetic and equilibrium binding properties depend on protein identity as well as particle surface characteristics and size.

Chavelas E. A., Zubillaga R. A., Pulido N. O., and Garcia-Hernandez E. (2006) Multithermal titration calorimetry: a rapid method to determine binding heat capacities. *Biophys Chem* **120,** 10-14.
Abstract: Herein a new method that allows binding DeltaCp to be determined with a single experiment is presented. Multithermal titration calorimetry (MTC) is a simple extension of isothermal titration calorimetry (ITC) that explicitly takes into account the thermal dependences of DeltaH and the binding constant. Experimentally, this is accomplished by performing a single stepwise titration with ITC equipment, allowing temperature re-adjustments of the system at intermediate states of the titration process. Thus, from the resulting multitherm, DeltaCp can also be determined. The experimental feasibility of MTC was tested by using the well-characterized lysozyme-chitotriose complex as a model system.

Chen A. and Wadso I. (1982) Simultaneous determination of Δ G, Δ H and Δ S by an automatic microcalorimetric titration technique. Application to protein ligand binding. *J Biochem Biophys Methods* **6,** 307-316.
Abstract: A methodological study has been made with a syringe titration unit attached to an LKB batch microcalorimeter. The precision and accuracy of the instrument assembly have been evaluated by neutralization reactions and by dilution of sucrose solutions. As an example, heat quantities on the order of 10 mJ accompanying the addition of 10 μliter titrant solution could be determined with an accuracy of better than 1%. A stepwise titration procedure was used to characterize the binding of indole-3-propionic acid to alpha-chymotrypsin. The following thermodynamic data were obtained (25 degrees C, acetate buffer, pH 5.80): Δ G0=-18.46 +/- 0.17 kJ X mol^{-1}, Δ H0=-15.26 +/- 0.20 kJ X mol^{-1}, Δ S0 = 10.85 +/- 1.21 JK-1 X mol^{-1}.

Chen A. and Wadso I. (1982) A test and calibration process for microcalorimeters used as thermal power meters. *J Biochem Biophys Methods* **6,** 297-306.
Abstract: A test and calibration process for microcalorimeters is described. The method has been developed with particular reference to instruments used for measurements of thermal power produced by suspensions of living cells. The process investigated is the hydrolysis of triacetin in imidazole/acetic acid buffer. The power levels are regulated by changing the buffer composition. The power will decrease slowly and very nearly linearly with time. Five test solutions, power levels 7-90 μW X ml^{-1}, have been characterized at 37 degrees C and one of them at 25 degrees C (13 μW X ml^{-1}). The power values for these reaction mixtures can be accurately calculated (+/- 0.5%) as a function of time during extended reaction periods, about 20 h or more.

Egawa T., Tsuneshige A., Suematsu M. and Yonetani T. (2007) Method for determination of association and dissociation rate constants of reversible bimolecular reactions by isothermal titration calorimeters. *Anal Chem* **79,** 2972-2978.
Abstract: The rate law equation for reversible bimolecular reactions, which are describable by association and dissociation rate constants (k1 and k-1), is not solvable to a plain formula under stoichiometric reaction conditions. Therefore, it is a general technique to observe such reactions under pseudo first-order conditions, which make the reactions a single-exponential process, and enable us to determine k1 and k-1 without any complicated iterative computations needed to analyze the same reactions under stoichiometric reaction conditions. However, the accelerated reaction rates under pseudo first-order conditions are not always favorable to the physicochemical tools employing a slow or medium response time, such as thermal analysis instruments. In this study, we have developed a simple non-iterative analytical method to determine k1 and k-1 of reversible bimolecular reactions under stoichiometric conditions on the basis of experimental data of isothermal titration calorimetry (ITC), which is generally used to determine thermodynamic parameters rather than kinetic constants. Our method is principally based on the general principle of chemical bindings caused along with the titration processes, that is, the chemical relaxation kinetics, which had been hitherto considered in the analysis on the ITC data.

Fanghanel J., Wawra S., Lucke C., Wildemann D., and Fischer G. (2006) Isothermal calorimetry as a tool to investigate slow conformational changes in proteins and peptides. *Anal Chem* **78,** 4517-4523.
Abstract: A new calorimetric method has been developed to follow the time course of slow conformational changes during the refolding of denatured proteins. The method is based on the ability of isothermal titration calorimeters (ITC) to detect small amounts of heat continuously over a minute to an hour time range without being disturbed by baseline drift. We benchmarked the method on the basis of the slow

kinetic phases resulting from prolyl cis/trans isomerization of oligopeptides. Using this method, the simultaneous investigation of the kinetics and thermodynamics of slow phases in the refolding of GdmCl-denatured RNase A by single jump techniques was performed. Time traces of heat production in the presence of a peptidyl prolyl cis/trans isomerase support the classical model of rate-limiting prolyl trans to cis isomerizations in the folding reactions of RNase A. However, we also observed that, unlike prolyl cis/trans isomerizations in oligopeptides, those found in RNase A refolding are highly exothermic. It appears that coupling between slow prolyl trans to cis isomerization and relocation of remote backbone segments increases the number of contacting sites during formation of the native protein. The results demonstrate that calorimetrically monitored folding kinetics will be of relevance in the detection of otherwise silent folding events.

Freyer M. W. and Lewis E. A. (2008) Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions. *Methods Cell Biol* **84,** 79-113.
Abstract: Isothermal titration calorimetry (ITC) is now routinely used to directly characterize the thermodynamics of biopolymer binding interactions and the kinetics of enzyme-catalyzed reactions. This is the result of improvements in ITC instrumentation and data analysis software. Modern ITC instruments make it possible to measure heat effects as small as 0.1 microcal (0.4 microJ), allowing the determination of binding constants, K's, as large as 10(8) - 10(9)M(-1). Modern ITC instruments make it possible to measure heat rates as small as 0.1 microcal/sec, allowing for the precise determination of reaction rates in the range of 10(-12) mol/sec. Values for K(m) and k(cat), in the ranges of 10(-2) - 10(3) microM and 0.05 - 500 sec(-1), respectively, can be determined by ITC. This chapter reviews the planning of an optimal ITC experiment for either a binding or kinetic study, guides the reader through simulated sample experiments, and reviews analysis of the data and the interpretation of the results

Garcia-Fuentes L., Baron C., and Mayorga O. L. (1998) Influence of dynamic power compensation in an isothermal titration microcalorimeter. *Anal Chem* **70,** 4615-4623.
Abstract: A theoretical analysis in Laplace's transformed domain based on a power balance represents a suitable model for an isothermal titration calorimeter with dynamic power compensation, designed and implemented in our laboratory. A rigorous calibration of the injection system and the calorimetric response was also made. Using electrically generated heat pulses, two different time constants have been determined from the calorimetric transfer function and assigned to the physical parts of the calorimeter. The same was done for a protein-ligand interaction. The binding of 2'-CMP to ribonuclease A at low and high ionic strengths was used to check the apparatus and the results were compared with those obtained by other authors (Wiseman, T.; Williston, S.; Brandts, J.F.; Lung-Nan, L. Anal. Biochem. 1989, 179, 131-137). In this case, the analysis showed a different time constant for the heat source. Independently of the nature of the heat source, the calorimetric time constants obtained while working under compensation are always smaller than those corresponding to a noncompensated system. The improvement of the calorimetric response introduced by dynamic power compensation is thus explained in terms of the reduction of the time constants characteristic of the calorimeter. This theoretical model can be used to predict the shape of the thermogram for any given reaction of either known or supposed thermodynamic parameters. Therefore, the calorimetric study is extended to the other nucleotides, 2'-UMP and 5'-dUMP, which have not hitherto been reported in the literature.

Harrous M. E. and Parody-Morreale A. (1997) Measurement of biochemical affinities with a Gill titration calorimeter. *Anal Biochem* **254,** 96-108.
Abstract: A Gill titration calorimeter is evaluated as an instrument to determine in one experiment the equilibrium constant and the enthalpy change of a biochemical reaction. The dimensionless parameter kc (the product of the association equilibrium constant and the concentration of the reagent to be titrated; Wiseman et al., Anal. Biochem. 179, 131-137, 1989) is used to analyze the instrument performance. The analysis of simulated titration data corresponding to a simple model case shows that association equilibrium constants in the 10^{2}-10^{7} M^{-1} range may be determined when the kc parameter is between 1 and 1000. In addition we use a Monte Carlo approach to estimate the precision in the thermodynamic parameters of the reaction under study. The relative precision in the calculated constants ranges from 3 to 80% depending on the macromolecule concentration and kc value in the experiment. These results were checked with the study of the reactions of beta-trypsin with its inhibitor and ribonuclease A with cytidine 2'-monophosphate and cytidine 3'-monophosphate.

Hong L., Bush W. D., Hatcher L. Q. and Simon J. (2008) Determining thermodynamic parameters from isothermal calorimetric isotherms of the binding of macromolecules to metal cations originally chelated by a weak ligand. *J Phys. Chem B* **112,** 604-611.
Abstract: An accurate data analysis method for determining stoichiometry and thermodynamic parameters from isothermal titration calorimetry data for the binding of macromolecules to metal cations that are solubilized through an association with a weak ligand is presented. This approach is applied to determine the binding constant for the association of Cu(II) to the first 16 residues of the Alzheimer's amyloid beta peptide, Abeta(1-16) under conditions where Cu(II) is rendered soluble through weak binding to glycine. At pH 7.2 and 37 degrees C, a binding constant of 1.5 x 10(9) M-1 (Kd = 0.7 nM) is determined for the association of Cu(II) with Abeta(1-16)

Houtman J. C., Brown P. H., Bowden B., Yamaguchi H., Appella E., Samelson L. E., and Schuck P. (2007) Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: Application to adaptor protein complexes in cell signaling. *Protein Sci* **16,** 30-42.
Abstract: Multisite interactions and the formation of ternary or higher-order protein complexes are ubiquitous features of protein interactions. Cooperativity between different ligands is a hallmark for information transfer, and is frequently critical for the biological function. We describe a new computational platform for the global analysis of isothermal titration calorimetry (ITC) data for the study of binary and ternary multisite interactions, implemented as part of the public domain multimethod analysis software SEDPHAT. The global analysis of titrations performed in different orientations was explored, and the potential for unraveling cooperativity parameters in multisite interactions was assessed in theory and experiment. To demonstrate the practical potential and limitations of global analyses of ITC titrations for the study of cooperative multiprotein interactions, we have examined the interactions of three proteins that are critical for signal transduction after T-cell activation, LAT, Grb2, and Sos1. We have shown previously that multivalent interactions between these three molecules promote the assembly of large multiprotein complexes important for T-cell receptor activation. By global analysis of the heats of binding observed in sets of ITC injections in different orientations, which allowed us to follow the formation of binary and ternary complexes, we observed negative and positive cooperativity that may be important to control the pathway of assembly and disassembly of adaptor protein particles.

Kim W., Yamasaki Y., and Kataoka K. (2006) Development of a fitting model suitable for the isothermal titration calorimetric curve of DNA with cationic ligands. *J Phys Chem B Condens Matter Mater Surf Interfaces Biophys* **110,** 10919-10925.
Abstract: A novel curve fitting model was developed for the isothermal titration calorimetry (ITC) of a cationic ligand binding to DNA. The ligand binding often generates a DNA conformational change from an elongated random coil into a compact collapsed form that is referred to as "DNA condensation". The ligand binding can be classified into two regimes having different binding constants Ki, i.e., the binding to an elongated DNA chain with a binding constant K1 and with K2 that occurred during the conformational transition. The two-variable curve fitting models are usually bound by a strict regulation on the difference in the values of the binding constants K1 > K2. For the DNA condensation, however, the relationships for K1 and K2 are still unclear. The novel curve fitting model developed in this study takes into account this uncertainty on the relationship of the binding constants and is highly flexible for the two-variable binding constant system.

Langerman N. and Biltonen R. L. (1979) Microcalorimeters for biological chemistry: applications, instrumentation and experimental design. *Methods Enzymol* **61,** 261-286.

Li L., Dantzer J. J., Nowacki J., O'Callaghan B. J. and Meroueh S. O. (2008) PDBcal: a comprehensive dataset for receptor-ligand interactions with three-dimensional structures and binding thermodynamics from isothermal titration calorimetry. *Chem Biol Drug Des* **71,** 529-532.
Abstract: Compounds designed solely based on structure often do not result in any improvement of the binding affinity because of entropy-enthalpy compensation. Thermodynamic data along with structure provide an opportunity to gain a deeper understanding of this effect and aid in the refinement of scoring functions used in computational drug design. Here, we scoured the literature and constructed the most comprehensive hand-curated calorimetry dataset to date. It contains thermodynamic and structural data for

more than 400 receptor-ligand complexes. The dataset can be accessed through a web interface at http://www.pdbcal.org. The thermodynamic data consists of free energy, enthalpy, entropy and heat capacity as measured by isothermal titration calorimetry (ITC). The dataset also contains the experimental conditions that were used to carry out the ITC experiments. The chemical structures of the ligands are also provided. Analysis of the data confirms the existence of enthalpy-entropy compensation effect for the first time using strictly ITC data

Marini M. A., Evans W. J., and Berger R. L. (1985) Use of the twin-cell differential titration calorimeter for binding studies. I. EDTA and its calcium complex. *J Biochem Biophys Methods* **10,** 273-285.
Abstract: The use of a twin-cell differential titration calorimeter is described which utilizes small volumes (1-3 ml) of modest concentrations of materials (0.001-0.01 M) and yields data of good precision. Operation is controlled by a microprocessor which regulates and controls the addition of reagents and collects and displays the data as time, temperature in volts, and the pH. Corrections for the titration of water are applied to the potentiometric data, and the thermal data are corrected for the initial temperature-time baseline, the changes in heat capacity, and the heat loss (or gain) to the external environment. Finally, the thermal signal is corrected for the heat derived from the formation of water due to the free hydrogen or hydroxyl ions present. The corrected data as pH, groups titrated and Δ HT (kcal/mol) can then be used to obtain the parameters pK' and Δ Hi involved with the equilibria by curve-fitting the observed data. The system has been applied to the ionization of EDTA and its calcium complex. The ionization constants, the heats of ionization, the stoichiometry of binding and the heat of binding have been determined and demonstrated to be in agreement with published values.

Marini M. A., Evans W. J., and Berger R. L. (1986) The determination of binding constants with a differential thermal and potentiometric titration apparatus. II. EDTA, EGTA and calcium. *J Biochem Biophys Methods* **12,** 135-146.
Abstract: A differential pH and thermal titrimeter has been used to determine the ionization constants of EDTA and EGTA as well as their calcium complexes. The intrinsic heat of binding is a constant for the pH range 2-11.5 for both substances and is found to be -5.4 kcal mol for EDTA and -7.9 kcal mol-1 for EGTA. The binding constants evaluated by fitting to the potentiometric curves and expressed as the log are 10.25 and 11.0, respectively. These values compare reasonably well to those reported in the literature. We have proposed that the binding of calcium occurs even at acid pH based on the pH drop observed at pH 2 when calcium is added, the reversibility of the thermal and the potentiometric curves, the lack of hysteresis upon rapid titration, the constancy of the intrinsic heat of binding and fitting of the entire potentiometric curves using the appropriate binding constants.

Markova N. and Hallen D. (2004) The development of a continuous isothermal titration calorimetric method for equilibrium studies. *Anal Biochem* **331,** 77-88.
Abstract: A continuous isothermal titration calorimetry (cITC) method for microcalorimeters has been developed. The method is based on continuous slow injection of a titrant into the calorimetric vessel. The experimental time for a cITC binding experiment is 12-20 min and the number of data points obtained is on the order of 1000. This gives an advantage over classical isothermal titration calorimetry (ITC) binding experiments that need 60-180 min to generate 20-30 data points. The method was validated using two types of calorimeters, which differ in calorimetric principle, geometry, stirring, and way of delivering the titrant into the calorimetric vessel. Two different experimental systems were used to validate the method: the binding of Ba(2+) to 18-crown-6 and the binding of cytidine 2'-monophosphate to RNAse A. Both systems are used as standard test systems for titration calorimetry. Computer simulations show that the dynamic range for determination of equilibrium constants can be increased by three orders of magnitude compared to that of classical ITC, making it possible to determine high affinities. Simulations also show an improved possibility to elucidate the actual binding model from cITC data. The simulated data demonstrate that cITC makes it easier to discriminate between different thermodynamic binding models due to the higher density of data points obtained from one experiment.

Maskow T., Lerchner J., Peitzsch M., Harms H., and Wolf G. (2006) Chip calorimetry for the monitoring of whole cell biotransformation. *J Biotechnol* **122,** 431-442.
Abstract: Efficient control of whole cell biotransformation requires quantitative real-time information about the thermodynamics and kinetics of growth and product formation. Heat production contains such

information, but its technical application is restricted due to the high price of calorimetric devices, the difficulty of integrating them into existing bio-processes and the slow response times of established microcalorimeters. A new generation of chip or nanocalorimeters may overcome these weaknesses. We thus tested a highly sensitive chip calorimeter for its applicability in biotechnological monitoring. It was used to monitor aerobic growth of suspended and immobilized Escherichia coli DH5alpha DSM 6897 and anaerobic growth of suspended Halomonas halodenitrificans CCM 286(T). The chip data corresponded well with enthalpy balance calculations and measurements with a conventional calorimeter, indicating the applicability of the chip calorimeter for bio-process control.

McKinnon I. R., Fall L., Parody-Morreale A., and Gill S. J. (1984) A twin titration microcalorimeter for the study of biochemical reactions. *Anal Biochem* **139,** 134-139.
Abstract: A small-volume (200 µliter) titration calorimeter of high sensitivity (1 mu cal) has been developed for the purpose of studying biochemical reactions where the amounts of material are limited to a few nanomoles. High sensitivity is achieved by calorimetric twining , use of glass cells, elimination of vapor space, effective low-energy stirring, and reduction of measurement time. The calorimeter operates using the heat conduction principal with computer-controlled electrical compensation, which reduces the measurement time of each point from 10 to 3 min. This reduction in time is accompanied by a corresponding increase in the precision of measurement. The use of the calorimeter is demonstrated by a measurement of the heat of oxygenation of hemocyanin.

Mizoue L. S. and Tellinghuisen J. (2004) The role of backlash in the "first injection anomaly" in isothermal titration calorimetry. *Anal Biochem* **326,** 125-127.

Mokdad A., Nissen M., Satterlee J. D. and Larsen R. W. (2007) Evidence for fast conformational change upon ligand dissociation in the HemAT class of bacterial oxygen sensors. *FEBS Lett* **581,** 4512-4518.
Abstract: Here we report the results of transient absorption and photoacoustic calorimetry studies of CO photodissociation from the heme domain of the bacterial oxygen sensor HemAT-Bs. The results indicate that CO photolysis is accompanied by an overall DeltaH of -19 kcal mol(-1) and DeltaV of +4 ml mol(-1) as well as a red-shifted kinetic difference spectrum all occurring in <50 ns. Analysis of the DeltaH/DeltaV reveals that a conformational change takes place with a DeltaH(conf) of -40 kcal mol(-1) and DeltaV(conf) of -22 ml mol(-1). These thermodynamic changes are consistent with an increase in the solvent accessible surface area of the protein upon ligand dissociation, as observed in the X-ray structure of the ferric CN-bound and CN free forms of HemAT-Bs.

Nakamura S. and Kidokoro S. (2004) Isothermal acid-titration calorimetry for evaluating the pH dependence of protein stability. *Biophys Chem* **109,** 229-249.
Abstract: A new method, which can be called as isothermal acid-titration calorimetry (IATC), was proposed for evaluating the enthalpy of protein molecules as a function of pH using isothermal titration calorimetry (ITC). This measurement was used to analyze the acid-denaturation of bovine ribonuclease A. The enthalpy change by acid-denaturation of this protein was estimated as 310 kJ/mol at pH 2.8 and 40 degrees C. This value agreed well with the enthalpy change obtained by differential scanning calorimetry. The midpoint pH and proton binding-number difference observed by IATC agreed well with those of the acid transition of the three-dimensional structure monitored by circular dichroism spectrometry. The van't Hoff enthalpy of the transition was derived from the temperature dependence of the midpoint pH and the proton binding-number difference. It agreed well with the calorimetric enthalpy change directly observed by IATC, strongly indicating that there was no stable intermediate state during the acid transition of this protein.

Nakamura S. and Kidokoro S. (2005) Direct observation of the enthalpy change accompanying the native to molten-globule transition of cytochrome c by using isothermal acid-titration calorimetry. *Biophys Chem* **113,** 161-168.
Abstract: The enthalpy change accompanying the reversible acid-induced transition from the native (N) to the molten-globule (MG) state of bovine cytochrome c was directly evaluated by isothermal acid-titration calorimetry (IATC), a new method for evaluating the pH dependence of protein enthalpy. The enthalpy change was 30 kJ/mol at 30 degrees C, pH 3.54, with 500 mM KCl. The results of the global analysis of the temperature dependence of the excess enthalpy from 20 to 35 degrees C demonstrated that the N to MG

transition is a two-state transition with a small heat capacity change of 1.1 kJ K^{-1} mol^{-1}. The present findings were also indicative of the pH dependence of the enthalpy and the heat capacity of the MG state, -13 kJ mol^{-1} $pH^{-1}$and -1.0 kJ K^{-1} mol^{-1} pH^{-1}, respectively, at 30 degrees C within a pH range from 2 to 3.

Saboury, A.A., Atri, M.S., Sanati, M.H., and Sadeghi, M. (2006) Application of a simple calorimetric data analysis on the binding study of calcium ions by human growth hormone. *J Thermal Anal Calor* **83**, 175-179.
Abstract: A simple graphical linear method was introduced for isothermal titration calorimetric data analysis in the protein-ligand interaction. The number of binding sites, the dissociation binding constant and the molar enthalpy of binding site can be obtained by using this new isothermal titration calorimetric data analysis method. The method was applied to the study of the interaction of human growth hormone (hGH) with divalent calcium ion at 27°C in NaCl solution, 50 mM. hGH has a set of three identical and independent binding sites for Ca 2+ . The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 52 μMand -17.4, respectively. Results obtained by this new calorimetric data analysis are in good agreement with results obtained using our previous method.

Tellinghuisen J. (2005) Statistical error in isothermal titration calorimetry: Variance function estimation from generalized least squares. *Anal Biochem* **343,** 106-115.
Abstract: The method of generalized least squares (GLS) is used to assess the variance function for isothermal titration calorimetry (ITC) data collected for the 1:1 complexation of Ba(2+) with 18-crown-6 ether. In the GLS method, the least squares (LS) residuals from the data fit are themselves fitted to a variance function, with iterative adjustment of the weighting function in the data analysis to produce consistency. The data are treated in a pooled fashion, providing 321 fitted residuals from 35 data sets in the final analysis. Heteroscedasticity (nonconstant variance) is clearly indicated. Data error terms proportional to q(i) and q(i)/v are well defined statistically, where q(i) is the heat from the ith injection of titrant and v is the injected volume. The statistical significance of the variance function parameters is confirmed through Monte Carlo calculations that mimic the actual data set. For the data in question, which fall mostly in the range of q(i)=100-2000mucal, the contributions to the data variance from the terms in q(i)(2) typically exceed the background constant term for q(i)>300mucal and v<10mul. Conversely, this means that in reactions with q(i) much less than this, heteroscedasticity is not a significant problem. Accordingly, in such cases the standard unweighted fitting procedures provide reliable results for the key parameters, K and DeltaH(degrees) and their statistical errors. These results also support an important earlier finding: in most ITC work on 1:1 binding processes, the optimal number of injections is 7-10, which is a factor of 3 smaller than the current norm. For high-q reactions, where weighting is needed for optimal LS analysis, tips are given for using the weighting option in the commercial software commonly employed to process ITC data.

Tellinghuisen J. (2004) Statistical error in isothermal titration calorimetry. *Methods Enzymol* **383,** 245-282.

Tellinghuisen J. (2004) Volume errors in isothermal titration calorimetry. *Anal Biochem* **333,** 405-406.

Tellinghuisen J. (2006) Van't Hoff analysis of K degrees (T): how good...or bad? *Biophys Chem* **120,** 114-120.
Abstract: Binding constant data K degrees (T) are commonly subjected to van't Hoff analysis to extract estimates of DeltaH degrees, DeltaS degrees, and DeltaCP degrees for the process in question. When such analyses employ unweighted least-squares fitting of lnK degrees to an appropriate function of the temperature T, they are tacitly assuming constant relative error in K degrees. When this assumption is correct, the statistical errors in DeltaG degrees, DeltaH degrees, DeltaS degrees, DeltaCP degrees, and the T-derivative of DeltaCP degrees (if determined) are all independent of the actual values of K degrees and can be computed from knowledge of just the T values at which K degrees is known and the percent error in K degrees. All of these statistical errors except that for the highest-order constant are functions of T, so they must normally be calculated using a form of the error propagation equation that is not widely known. However, this computation can be bypassed by defining DeltaH degrees as a polynomial in (T-T0), the coefficients of which thus become DeltaH degrees, DeltaCP degrees, and 1/2 dDeltaCP degrees/dT at T=T0. The errors in the key quantities can then be computed by just repeating the fit for different T0. Procedures for doing this are described for a representative data analysis program. Results of such

calculations show that expanding the T range from 10-40 to 5-45 degrees C gives significant improvement in the precision of all quantities. DeltaG degrees is typically determined with standard error a factor of approximately 30 smaller than that for DeltaH degrees. Accordingly, the error in TDeltaS degrees is nearly identical to that in DeltaH degrees. For 4% error in K degrees, the T-derivative in DeltaCP degrees cannot be determined unless it is approximately 10 cal mol-1 K-2 or greater; and DeltaCP degrees must be approximately 50 cal mol-1 K-1. Since all errors scale with the data error and inversely with the square root of the number of data points, the present results for 4% error cover any other relative error and number of points, for the same approximate T structure of the data.

Tellinghuisen J. (2007) Calibration in isothermal titration calorimetry: Heat and cell volume from heat of dilution of NaCl(aq). *Anal Biochem* **360,** 47-55.
Abstract: An isothermal titration calorimeter of the perfusion type (MicroCal model VP-ITC) is calibrated using the heat of dilution of NaCl in water. The relative apparent molar enthalpy function (L(varphi)) for NaCl(aq) varies strongly and nonlinearly with concentration in the low-concentration region (<0.2M) that is sampled easily and extensively in a single program of injections of NaCl solution into water. This nonlinearity makes it possible to calibrate with respect to two quantities: the measured heat and the active cell volume. The heat factor is determined with typical standard error 0.003; its value in the current case is 0.987. The cell volume factor is 0.93 but is quite sensitive to possible systematic errors in the temperature and in the literature values for L(varphi). Both correction factors are closely tied to the delivered volume from the injection syringe, which required a correction factor of 0.973, attributed to an instrumental gear ratio error. Temperature calibration of the instrument showed a small offset of 0.12K at the temperature 25 degrees C of the experiments, but the error increased to more than 1K at 46 degrees C. The experiments were not able to distinguish clearly between mixing algorithms that assume instantaneous mixing on injection and those that assume instantaneous injection followed by mixing; however, examination of these algorithms has revealed an error in a program widely used to analyze isothermal titration calorimetry data.

Tellinghuisen J. (2007) Optimizing experimental parameters in isothermal titration calorimetry: variable volume procedures. *J Phys. Chem B* **111,** 11531-11537.
Abstract: In the study of 1:1 binding, M + X right <==> 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 identical with DeltaH degrees [M]0, low, as well. Successful determination of K and DeltaH degrees 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, DeltaH degrees and the stoichiometry parameter n (often called site number) also become highly correlated and individually indeterminate. However, the product DeltaH degrees x n (identical with Hn) is well-determined, so if n is known from other information, both K and DeltaH degrees 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 Hn values, permitting determination of K to better than 10% and Hn 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 Ba2+/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.

Tellinghuisen J. (2008) Isothermal titration calorimetry at very low c. *Anal Biochem* **373,** 395-397.
Abstract: In the study of 1:1 binding, M + X right arrow over left arrow MX, isothermal titration calorimetry (ITC) can be used successfully at values of c=K[M](0) well below the value 1.0 that is often considered its lower limit. However, analysis of low-c ITC data may require freezing the stoichiometry parameter n, and that is thought to be prohibitive for biological systems, where n can be poorly known. Here it is noted that the least-squares estimates of the binding constant K are virtually independent of errors in n at low c, permitting reliable determination of K and, from its temperature dependence, DeltaH degrees and n, down to c=10(-4) or lower, ligand solubility permitting.

Tellinghuisen J. (2008) Stupid statistics! *Methods Cell Biol* **84,** 739-780.
Abstract: The method of least squares is probably the most powerful data analysis tool available to scientists. Toward a fuller appreciation of that power, this work begins with an elementary review of statistics fundamentals, and then progressively increases in sophistication as the coverage is extended to the theory and practice of linear and nonlinear least squares. The results are illustrated in application to data analysis problems important in the life sciences. The review of fundamentals includes the role of sampling and its connection to probability distributions, the Central Limit Theorem, and the importance of finite variance. Linear least squares are presented using matrix notation, and the significance of the key probability distributions-Gaussian, chi-square, and t-is illustrated with Monte Carlo calculations. The meaning of correlation is discussed, including its role in the propagation of error. When the data themselves are correlated, special methods are needed for the fitting, as they are also when fitting with constraints. Nonlinear fitting gives rise to nonnormal parameter distributions, but the 10% Rule of Thumb suggests that such problems will be insignificant when the parameter is sufficiently well determined. Illustrations include calibration with linear and nonlinear response functions, the dangers inherent in fitting inverted data (e.g., Lineweaver-Burk equation), an analysis of the reliability of the van't Hoff analysis, the problem of correlated data in the Guggenheim method, and the optimization of isothermal titration calorimetry procedures using the variance-covariance matrix for experiment design. The work concludes with illustrations on assessing and presenting results.

Turnbull W. B. and Daranas A. H. (2003) On the value of c: can low affinity systems be studied by isothermal titration calorimetry? *J Am Chem Soc* **125,** 14859-14866.
Abstract: Isothermal titration calorimetry (ITC) allows the determination of ΔG degrees, ΔH degrees, and ΔS degrees from a single experiment and is thus widely used for studying binding thermodynamics in both biological and synthetic supramolecular systems. However, it is widely believed that it is not possible to derive accurate thermodynamic information from ITC experiments in which the Wiseman "c" parameter (which is the product of the receptor concentration and the binding constant, Ka) is less than ca. 10, constraining its use to high affinity systems. Herein, experimental titrations and simulated data are used to demonstrate that this dogma is false, especially for low affinity systems, assuming that (1) a sufficient portion of the binding isotherm is used for analysis, (2) the binding stoichiometry is known, (3) the concentrations of both ligand and receptor are known with accuracy, and (4) there is an adequate level of signal-to-noise in the data. This study supports the validity of ITC for determining the value of Ka and, hence, ΔG degrees from experiments conducted under low c conditions but advocates greater caution in the interpretation of values for ΔH degrees. Therefore, isothermal titration calorimetry is a valid and useful technique for studying biologically and synthetically important low affinity systems.

Velazquez-Campoy A. (2006) Ligand binding to one-dimensional lattice-like macromolecules: Analysis of the McGhee-von Hippel theory implemented in isothermal titration calorimetry. *Anal Biochem* **348,** 94-104.
Abstract: The theory developed by McGhee and von Hippel for ligand binding to a one-dimensional lattice-like macromolecule provides a closed analytical form in the Scatchard representation. The application of such theory has been complicated by two facts: (1) it has been practically reduced to binding techniques, such as equilibrium dialysis, in which the partition between bound and free concentrations of all reactant species are directly accessible and experimentally determined, but infrequently applied to other binding techniques, such as calorimetry or spectroscopy, in which the direct observable is a magnitude proportional to the advance of the binding reaction monitored along the titration experiment, and (2) Scatchard analysis, developed as a quantitative graphical method, is currently outdated and used only qualitatively because of its weaknesses, limitations, and deficiencies. However, a general exact method for applying such theory to titration techniques in a correct and precise manner, without any limitation, can be delineated. In this article, the theory of cooperative ligand binding to linear lattice-like macromolecules has been implemented in isothermal titration calorimetry for the first time. This technique provides a complete thermodynamic characterization of ligand binding, but it has been barely used properly for this type of system. The description, the analysis of the formalism, and practical guidelines are presented, with considerations for experimental design and data analysis.

Velazquez-Campoy A., Goni G., Peregrina J. R., and Medina M. (2006) Exact analysis of heterotropic interactions in proteins: Characterization of cooperative ligand binding by isothermal titration calorimetry.

Biophys J **91,** 1887-1904.
Abstract: Intramolecular interaction networks in proteins are responsible for heterotropic ligand binding cooperativity, a biologically important, widespread phenomenon in nature (e.g., signaling transduction cascades, enzymatic cofactors, enzymatic allosteric activators or inhibitors, gene transcription, or repression). The cooperative binding of two (or more) different ligands to a macromolecule is the underlying principle. To date, heterotropic effects have been studied mainly kinetically in enzymatic systems. Until now, approximate approaches have been employed for studying equilibrium heterotropic ligand binding effects, except in two special cases in which an exact analysis was developed: independent binding (no cooperativity) and competitive binding (maximal negative cooperativity). The exact analysis and methodology for characterizing ligand binding cooperativity interactions in the general case (any degree of cooperativity) using isothermal titration calorimetry are presented in this work. Intramolecular interaction pathways within the allosteric macromolecule can be identified and characterized using this methodology. As an example, the thermodynamic characterization of the binding interaction between ferredoxin-NADP+ reductase and its three substrates, NADP+, ferredoxin, and flavodoxin, as well as the characterization of their binding cooperativity interaction, is presented.

Wiseman T., Williston S., Brandts J. F., and Lin L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal Biochem* **179,** 131-137.
Abstract: A new titration calorimeter is described and results are presented for the binding of cytidine 2'-monophosphate (2'CMP) to the active site of ribonuclease A. The instrument characteristics include very high sensitivity, rapid calorimetric response, and fast thermal equilibration. Convenient software is available for instrument operation, data collection, data reduction, and deconvolution to obtain least-squares estimates of binding parameters n, Δ H degree, Δ S degree, and the binding constant K. Sample through-put for the instrument is high, and under favorable conditions binding constants as large as 10^8 M^{-1} can be measured. The bovine ribonuclease A (RNase)/2'CMP system was studied over a 50-fold range of RNase concentration and at two different temperatures. The binding constants were in the 10^5 to 10^6 M-1 range, depending on conditions, and heats of binding ca. -15,000 cal/mol. Repeat determinations suggested errors of only a few percent in n, Δ H degree, and K values over the most favorable concentration range.

Xu J., Reiserer R., Tellinghuisen J., Wikswo J. P. and Baudenbacher F. J. (2008) A microfabricated nanocalorimeter: design, characterization, and chemical calibration. *Anal Chem* **80,** 2728-2733.
Abstract: A microfabricated titration calorimeter having nanowatt sensitivity is presented. The device is achieved by modifying a commercial, suspended-membrane, thin-film thermopile infrared sensor. Chemical reactions are studied by placing a 50.0 nL droplet of one reagent directly on the sensor and injecting nanoliter droplets of a second reagent through a micropipette by means of a pressure-driven droplet injector with 1% reliability in volume delivery. External thermal noise is minimized by a two-layer thermal shielding system. Evaporation is prevented by positioning the micropipette through a tiny hole in a cover glass, sealed by a drop of oil. The device is calibrated using two acid-base reactions: H2SO4 + HEPES buffer, and NaOH + HCl. The measured power sensitivity is 2.90(4) V/W, giving a detection limit of 22 nW. The 1/e time constant for a single injection is 1.1 s. The day-to-day power sensitivity is reproducible to approximately 2%. A computational model of the sensor reproduces the power sensitivity within 10% and the time constant within 20%. For a 50 nL sample and 0.8-1.5 nL titrant injection volumes, the heat uncertainty of 44 nJ corresponds to a 3sigma detection limit of 132 nJ, or the binding energy associated with 2.9 pM of IgG-protein A complex

Yang Y., Zhu J., Liu Y., Shen P., and Qu S. (2005) Microcalorimetry is a sensitive method for studying the effect of nucleotide mutation on promoter activity. *J Biochem Biophys Methods* **62,** 183-189.
Abstract: Microcalorimetric method was successfully used to study the effect of nucleotide mutations on promoter activity and identify the important nucleotide necessary for the promoter function in Escherichia coli. The thermokinetic parameters, such as k, I and IC(50), were calculated from the metabolic power-time curves obtained by microcalorimetric measurement using the TAM air Isothermal Microcalorimeter (manufactured by Thermometric AB company of Sweden). Analysis of these data revealed that different nucleotide mutations in -10 box sequence of RM07 fragment had different effect on the promoter activity. Our research also suggest that the microcalorimetric method is a very sensitive and easily performed method for investigation of promoter mutation.