Pure & Appl. Chem., Vol.52, pp.479-497. 0033-4545/80/0201-0479 $02.00/0
Pergamon Press Ltd. 1980. Printed in Great Britain.

SCANNING MICROCALORIMETERS FOR STUDYING MACROMOLECULES

Peter L. Privalov

Institute of Protein Research, Academy of Sciences of the USSR,
142292 Poustchino, Moscow Region, USSR

Abstract - Principles of scanning microcalorimeter design, their basic characteristics and microcalorimetric data processing in studying biological macromolecules in solution are discussed.

INTRODUCTION

In the mid-fifties a number of problems arose which could not be solved without measuring small heat effects in heated samples, i.e. without measuring slight changes of the heat capacity in a broad temperature range. The problems were connected not only with the study of materials difficult to obtain, but also with the study of diluted solutions. The latter task was even more important and complicated. Indeed, in this case the small heat capacity effect of the solute has to be measured on the greatly prevailing background of the solvent heat capacity. At the same time, while the expenditure of rare materials is only a question of economy, the concentration of solution is a crucial factor in obtaining reliable results. But in a dilute solution the effect which must be measured is also small, e.g. heat capacity contribution of a macromolecule in a 0.3% water solution amounts to only one thousandth of the total heat capacity.

The first sensitive instruments capable of measuring small heat effects in heated liquids appeared about fifteen years ago (1-4). About at the same time the instruments were also created for measuring heat absorption at heating of small samples (5,6). Extremely high sensitivity of all these instruments was achieved by a combination of certain techniques which will be considered below. Among them differential measurement at continuous heating or scanning over temperature scale was most essential new achievement.

The possibilities of differential scanning microcalorimetry have been discussed by both J. Sturtevant and myself at different conferences and in review papers (7-14). However little has been mentioned about the method per se, the technics of scanning calorimetry. The reason was that before discussing the technical aspect of this new method, it was necessary to demonstrate its real possibilities and to attract the attention of a wide field of investigators who had a rather vague idea of the value of calorimetric information. Now the situation is different: scanning microcalorimetry is in the focus of attention and it is timely to discuss the technical aspect and to make some forecasts taking into account the rapidly growing requirements demanded of these instruments.

GENERAL PRINCIPLES OF SCANNING MICROCALORIMETRY TECHNICQUES

Scanning

The advantages of continuous measurements at continuous heating of samples over a classical step-wise method of determination of heat capacity are evident. The continuous function is much more informative than a discrete set. But even more essential is that at continuous heating all the calorimetric system is in a steady state and its automation and control is much easier and can be performed with a higher accuracy.

However, continuous heating leads to principal complications. Indeed, for step-wise measurements it is possible to wait for the establishment of a complete thermal equilibrium, while for continuous heating this is impossible and measurements are taken without complete equilibrium in the system. There are two reasons of non-equilibrium: one is connected with the finiteness of heat conductivity of the object, and the other with the finiteness of the rate of temperature-induced processes. It is clear that the deviation from the equilibrium will increase with an increase of the heating rate.

In the case of microcalorimeters it is more or less easy to choose such a design of a calorimetric cell which would provide sufficiently small temperature differences within the object at heating, since the volume of the object itself is small. Indeed, we can say that only small objects permit passing to continuous heating. For in the absence of a stirrer, when heat conductivity is the only way of equalizing the temperature, the thickness of the heated layer of the sample is of paramount importance. But reducing the volume can be efficient only to a certain limit because it leads to an increase of the relative error in determining the amount of the sample. A further decrease of the thickness of the heated layer is possible only either by using a special internal heater with an extended radiator (as in the first instruments, Ref. 1-4) or by using cells with an extended surface which serves as a heater. Thus the cells do not have the minimal ratio of the surface area to the volume, as found in traditional heat-capacity calorimeters.

Differential method of measuring

A second distinguishing feature of heat-capacity microcalorimeters is that these instruments do not measure the absolute heat capacity of the object. But the difference heat capacity against some standard can be determined with higher accuracy. For evaluating the heat-capacity difference at each point in a temperature range, the standard sample and studied sample are heated simultaneously and in the same conditions. Therefore such calorimeters are always twin: they have two identical calorimetric cells, one with the standard (reference cell) and the other with the object (measuring cell). The cells are positioned in the most symmetrical way to each other and to the surroundings so that their heating conditions are maximally similar (Fig. 1). However, in reality it is

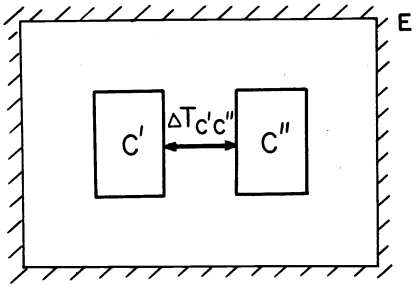

Fig. 1. Twin calorimeter with a reference (C') and measuring (C") cells and a temperature-sensitive element ($\Delta T_{C'C''}$) enclosed in environment with constant temperature E.

impossible to achieve complete similarity in the cells. In practice the difference heat capacity is determined not by the difference in heating the object and the standard which are loaded in the measuring and reference cells, but by the difference in heating the object and the standard which are placed one after the other into the same measuring cell, while the reference cell is always loaded by the standard. Thus, the difference heat capacity is determined in two stages: at first both cells are filled with the standard and the so-called "base line" of the instrument is determined, then the standard is replaced in the measuring cell by the object. By a comparison of two recordings we can determine the difference between the object and the standard, loaded in the same cell, and thus eliminate the contribution of non-identity of cells. Naturally, the higher the sensitivity of the instrument, the more pronounced becomes the non-identity of the cells, i.e. the more complicated is the base line which in highly sensitive instruments is seldom straight and horizontal.

Evidently the closer is the heat capacity of the standard to that of the object, the more accurately they can be compared. In studying solutions the most suitable standard liquid is undoubtedly the solvent, since in this case the difference in heat capacities is stipulated directly by the solute and, consequently, it is just this difference that yields information on the solute.

Compensation of heat effect

There are two basic methods for measuring the difference between heat capacities of the measuring and reference cells. The first consists in measuring the difference in temperature of the cells heated by the same power. The second consists in measuring the difference of powers necessary for heating both the cells with the same rate, i.e. it consists in determining the power which compensates the difference of the cells by heat capacity. However, in the first case we also have a compensation by the heat flow from the surroundings. This compensation is passive in contrast to the active compensation by the control-

led heating. The disadvantage of the first method is in its slowness - stationary heat flow is achieved with great delay, and also in this case the measured effect is strongly affected by the thermal contact of the cells. The advantage of the active compensation is that the heat effect is measured directly and not through the parameter which is assumed to be proportional to it. Therefore the first method is mainly used in thermographs for qualitative analysis, whereas in microcalorimetry, especially in precision microcalorimetry, active compensation is generally used.

There are several methods of compensation in scanning microcalorimetry:
(a) Compensation by direct current supplied to a special heater (Fig. 2).

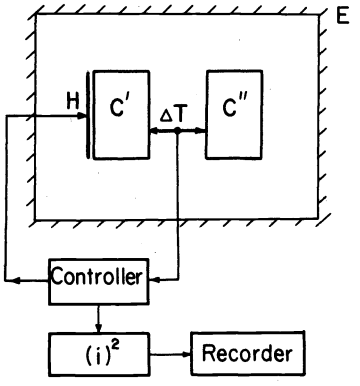

Fig. 2. Scanning calorimeter with compensation of the heat effect by direct current supplied to a special heater (H).

Since the power is proportional to the square of the current value ($w = ri^2$), it is necessary to register the squared current value, i.e. in this case the instrument must have a special device which will perform this operation automatically. Thus the simplicity of this method is only apparent (Ref. 4).
(b) Compensation by direct current supplied to the main heaters of both the cells. This method is also known as symmetrical compensation, since in each heater a current of opposite direction is supplied, decreasing the heat release power in one cell and increasing it in the other (see Fig. 3). In this case

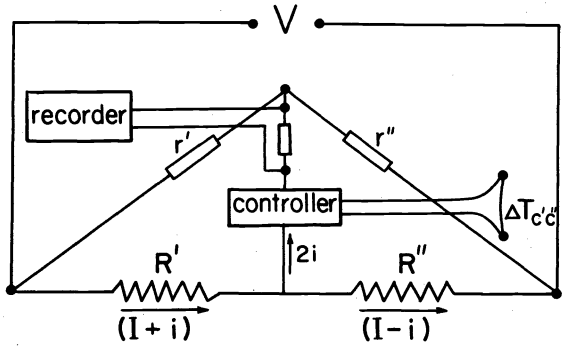

Fig. 3. Scheme of symmetric compensation by direct current supplied to the main heaters with the resistance R' and R".

the power in the first and the second cells is:

$$w' = R' (I + i)^2 \quad (1)$$

$$w'' = R'' (I - i)^2 . \quad (2)$$

The difference in power at equal resistance of the heaters (R' = R" = R) is:

$$\Delta w = w' - w'' = R [(I + i)^2 - (I - i)^2] = 4RIi . \quad (3)$$

Thus the difference power is a linear function of the compensating current which is very important for a feedback control. Method (b) undoubtedly has great advantages over method (a). Yet its disadvantage is that feedback here depends on the main current of heating the cells, I. As a result, the scale of

the instrument changes with a change of the heating rate.

Symmetrical compensation was first described by Privalov et al. (Ref. 1, for details see Ref. 15) and independently by Watson & O'Neil (6). Due to convenience and simplicity it was widely used both in precision scanning microcalorimeters (DASM-1M) and in instruments for thermal analysis (DSC Perkin-Elmer).

(c) Compensation by rectangular impulses of regulated frequency fed into a separate heater of one of the cells.

In this case:

$$\Delta w = ri^2 \tau \gamma \qquad (4)$$

where τ is the duration of a rectangular impulse of i current, γ is the frequency of impulses determined by a transducer of the imbalance signal into frequency. In this case the frequency γ is proportional to the compensating power. This method of compensation was first successfully used in microcalorimetry by Alberts (16).

(d) Compensation by rectangular constant-frequency impulses of regulated duration.

In this case:

$$\Delta w = ri^2 \gamma \tau \qquad (5)$$

where τ is the duration of an impulse adjusted by the transducer of the imbalance signal into the impulse duration. Here a registered value is the electrical parameter directly proportional to τ. In scanning calorimeters it was first used in the instrument DASM-4.

The advantage of compensation by the impulse current over the compensation by constant current consists in the independence of the compensation power of the heating rate. Therefore in this case it is not necessary to calibrate the instrument at each heating rate.

Thermosensors

Thermosensors detecting the temperature difference are among the most important elements of any calorimeters since their sensitivity determines to a great extent the overall sensitivity of the instrument.

Thermosensors used in calorimetry can be divided into three groups: thermoresistors, thermoelectric sensors and vibrators. In scanning microcalorimetry all these elements were used but not all of them have proved suitable for highly sensitive and precision instruments.

Let us consider the basic requirements to thermosensors for the precision scanning microcalorimeters. First of all they should measure small temperature difference, their readings should be stable and independent of time, temperature, and the number of heating cycles. The elements should have a low heat conductivity but good thermal contact with the body in which the temperature is detected. Their dimensions should be small and their heat capacity should be low. Finally, the heat production by the measuring elements should be insignificant. Evidently it is not easy to satisfy simultaneously all these requirements. At present thermoelectric sensors seem to be the most suitable for precision microcalorimetry. Of these semiconductor thermopiles are undoubtedly the most sensitive. However, their great disadvantage is their high heat capacity. Therefore up to the present time they have not been effectively used in precision scanning microcalorimetry where metal thermopiles are preferred. In the highly sensitive precision scanning microcalorimeter DASM-1M manufactured by the Academy of Sciences of the USSR, a 200 junction chromel-constantan thermopile is used which provides the sensitivity of 10^{-2} V/K (Ref. 17).

Heating of cells

The main requirement for heating the cells is the constant heating rate in the whole operational range of temperatures. If this requirement is not satisfied, it becomes difficult to correlate the observed effects with the real heat capacity change of the object.

Heating of the cells at a strictly constant rate in a wide range of temperatures is not so simple. First of all the heating rate is strongly influenced by the surrounding temperature. If this temperature is fixed, the heating rate will decrease linearly with temperature since heat losses of the cells will grow proportionally to the temperature difference of the cells and the surroundings. Hence it is clear that to make the heating rate constant at a constant surrounding temperature, the heating power of the cells should increase according to a certain program. However, it is not easy to program a linear increase of heating power (especially taking into account its squared dependence on the current value). Therefore linear heating is achieved by

programming the heating process using a linearly increasing voltage and adjusting the heating power so that the electrical signal for the cell temperature is equal to this increasing voltage (6). This method of linear heating was used in the DSC Perkin-Elmer calorimeter. The advantage of this method is that it permits not only linear heating with different rates, but also a linear cooling. A disadvantage of the method is that the heating power undergoes a manyfold change upon heating the system. Since it is impossible to produce absolutely identical cells and heaters, a change in the main heating current leads to great deviations between temperatures of both cells and distorts the base line. Therefore this type of instrument cannot be precise and is used mainly in applied studies.

From the above it follows that high sensitivity can be achieved only in instruments with constant basic heating power, i.e. in instruments where heat losses do not change at heating. This is possible only in the instruments where the surrounding temperature changes simultaneously with heating of the cells. Here we have two main approaches:

a) The surrounding temperature determines the temperature of the cells. In this case the cells are heated by a heat flow from the surroundings whose temperature changes at a constant rate. To achieve this, the cell surrounding is enclosed into an adiabatic shell with regulated temperature (Fig. 4).

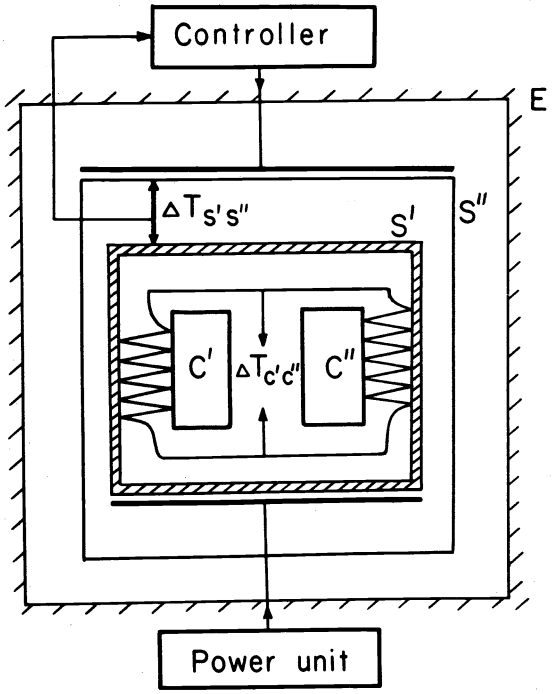

Fig. 4. Heat-flow scanning calorimeter with a variable temperature of the closest surrounding of the cells (S') and the controlled adiabatic shield (S").

This method of heating the cells was used in the calorimeter of Ross & Goldberg (18). The use of this method of heating the cells requires good thermal contact of the cells with the surrounding. At the same time, it is advisable that this contact be realized by a thermopile. In this case the heat flow through the thermopiles would generate an electromotive force proportional to the heat flow. Therefore in such calorimeters it is not the temperature differences between the cells that are measured but the difference between heat flows to the cells, as shown in Fig. 4, that is why these instruments are usually called "heat-flow scanning microcalorimeters".

b) The surrounding temperature is determined by the cell temperature. In this case the cells are heated by the constant current at a definite rate. The controller monitors heating of the surrounding (see Fig. 5). The surrounding temperature may be either equal (the adiabatic heating regime) or lower by a certain value (the cooling regime) than the temperature of the cells. Instruments of this type are usually called "adiabatic scanning microcalorimeters", although they operate not always in strictly adiabatic conditions. To decrease the influence of inaccuracy in adjusting the shields, vacuum insulation and additional water jackets were used in the first adiabatic instruments (see Fig. 6). In later designs a special multilayer system of shields was used in

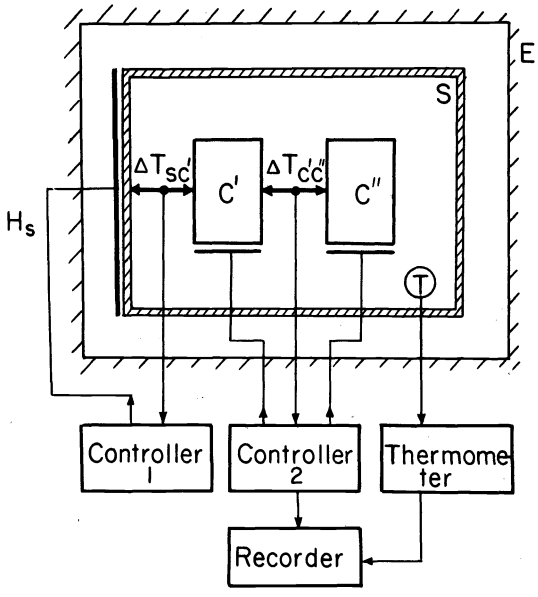

Fig. 5. Adiabatic scanning calorimeter with the cells heated at a constant rate and controlled temperature of the surrounding (S).

which particular attention was paid to symmetry (17). This system, called "sombrero", is shown in Fig. 7. Here the cells are enclosed into an adiabatic shell consisting of two identical hemispherical shields clamped to each other

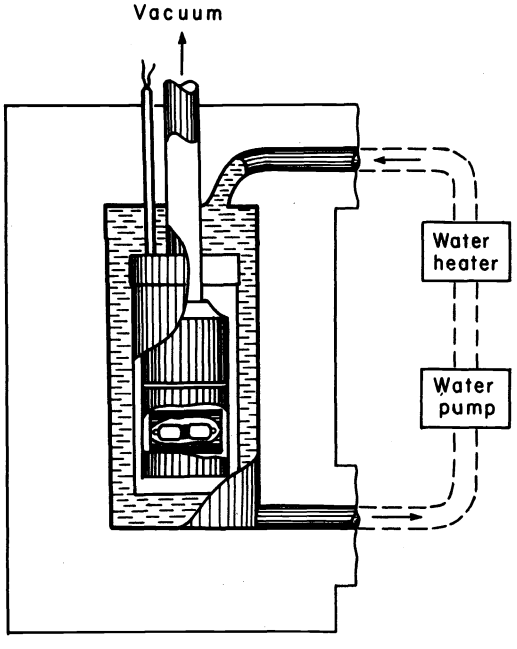

Fig. 6. Adiabatic scanning microcalorimeter with vacuum insulation and water jacket with controlled temperature. The twin cell is located within system of three shields (Ref. 15).

by rims. All connections and wires to the cells are laid in radial grooves between the rims. The equatorial plane of the shell is vertical, which is essential to achieve identical conditions for both halves of the shell.

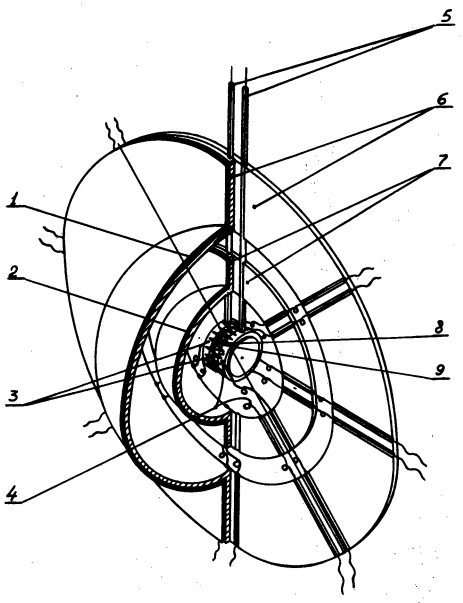

Fig. 7. System of adiabatization of the scanning microcalorimeter DASM-1M. 1,2 - internal and external adiabatic shells with heaters; 3 - calorimetric cells; 4 - shell thermosensor; 5 - capillary inlets; 6,7 - rims; 8 - thermopile; 9 - cell heater.

For accurate adjustment of the temperature of the adiabatic shell it is significant that its thermal inertia be as small as possible, i.e. the shield must be as thin as possible. However, a thin shield can give rise to surface inhomogeneities of temperature, in particular when supplying high power. To decrease the power fed to the shield and to make it constant, the shell is enclosed into a second shell. The temperature of the second shell is also adjusted by the controller system so that it is equal to that of the internal shell.

Though the construction of adiabatic calorimeters is more complicated than that of the non-adiabatic ones, this complexity justifies itself: the highest sensitivity has been achieved in adiabatic scanning microcalorimeters. They include instruments described by Gill & Beck (3) and Danford et al. (4) and all the instruments designed by Privalov.

Loading of the sample

One of the most difficult problems in precision microcalorimetry is the loading of the studied sample into the calorimeter. The complexity of this problem is due to a number of circumstances.

The first is connected with a calibration of the mass or of the volume of the sample studied. For the differential method of measurement it is important not only to know the precise amounts of the sample studied and the standard sample within the cells, but it is necessary to have strictly identical amounts of the samples. Evidently, if the measuring accuracy of the instrument is 10^{-4} cal/K, the accuracy of loading the cells with the sample and the standard should not be lower than 10^{-4} g. It is not easy to ensure such an accuracy, not only because of the errors in weighing the samples, but also due to an evaporation at the sealing procedure.

The second is connected with the problem of free space within the cell. It is clear that hermetic cells cannot be completely filled with the sample because of the thermal expansion of the sample at heating. But in the presence of free space in a cell, there will be also an evaporation effect upon heating which is different for the studied and the standard samples, since their vapour pressure cannot be identical. Though the vapour pressure does not differ greatly, the difference in the heat effect of evaporation can be significant due to the large evaporation enthalpy.

The third is the problem of the removal of cells (or more exactly, containers or ampoules enclosed in the cells) from the calorimeter for loading a new sample. After their removal it is practically impossible to reestablish the original conditions, because of the contamination of the surface, change in thermal contacts between the container and the cell, disturbance of electric contacts, etc.

As a result, scanning calorimeters with the removable containers cannot provide high accuracy in determining the differential heat capacity. In other words, such instruments cannot be precise in spite of their sufficiently high sensitivity. Thus, these instruments were suitable only for qualitative studies of the temperature dependence of heat capacities, but not for determining partial heat capacities and their dependence on temperature.

The development of a undismountable calorimetric system with cells which are completely filled by the sample was of crucial importance in changing the

situation (19). The idea of the system was that if the temperature of the cells and the surroundings is similar (i.e. the conditions are adiabatic), the cells can be connected with the surroundings by capillaries, and the whole volume of the cells and the capillaries can be filled with the sample studied or the standard sample, i.e. by the solution and the solvent (see Fig. 8). Under these conditions, the energy transfer from both the cells into the surroundings will

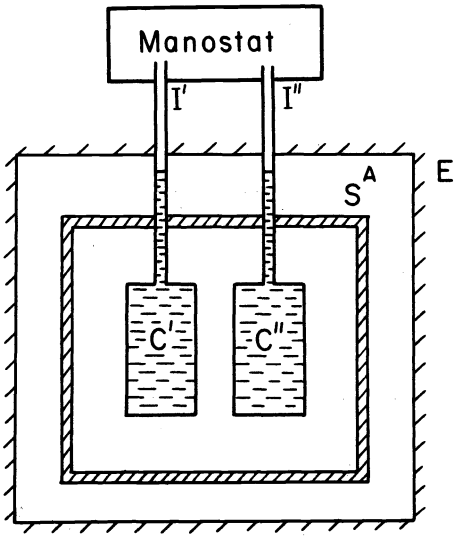

Fig. 8. Undismountable calorimetric block with the cells completely fillied by samples through the capillary inlets.

be practically the same, since the thermal expansion coefficients of the diluted solution and the solvent are very similar. The measuring volume in such cells is determined by the point of contact of capillaries with the adiabatic shell which, due to high thermal conductivity, acts as a thermal shunt limiting the measuring volume. Although this boundary is not clearly defined geometrically because of heat conductivity of the capillary walls and the liquid filling it, yet due to the similarity of the solution heat conductivity and that of the solvent, this boundary for the solution and the solvent will be very much the same. Therefore, to load the cells with the same amounts of the sample studied and the standard sample, it is necessary to just fill completely the cell volume and the inlet capillary volume up to above the contact point with the thermal shunt, i.e. the adiabatic shell. The principal difference of taking measurements by instruments with a cell completely filled and the sealed cells is that in the first case we determine heat capacity of the sample with a given volume and not with a given mass.

The main problem of loading the sample into this type of calorimeter consists in the complete filling of the volume without bubbles, since even microscopic bubbles may lead to an error in the heat capacity. To exclude gas bubbles from the measuring volume, a constant extra pressure is applied to the external inlet of the capillaries. 1.5 atm is sufficient to dissolve all the microscopic bubbles within the cell and to prevent their formation at heating up to 120°C. Such a very simple method almost completely eliminates measurement failures due to inaccurate filling and degassing and yields a high reproducibility of microcalorimetric experiments.

The undismountable calorimetric block with a two-layer adiabatic shell designed in our laboratory was already given in Fig. 7 (for details see Ref. 17). This block allowed one to obtain for the first time a high reproducibility of the base line at refilling the cells. With this block as a basis, the Special Bureau of Biological Instrumentation of the Academy of Sciences of the USSR has produced in 1974 a precision scanning microcalorimeter DASM-1M.

Capillary microcalorimeters

As has been noted, the conventional point of view for the advantage of the minimal ratio of the cell surface to its volume has had to be revised in microcalorimetry. This is due to the fact that the sample in microcalorimetry is heated mainly from the cell surface. Thus, the greater is the surface of the same volume, the lower are the temperature gradients in the heated sample, and consequently, the higher is the rate of its heating which allows to gain in sensitivity. These considerations, together with the stated above on the loading of the sample into the cell, enabled us to conclude that the cells from

capillary tubes are the most advantageous. Such cells are easier washed and filled without bubbles than any others. Moreover, much higher pressure can be applied to a capillary cell, and thus the upper limit of working temperatures for a capillary cell can be significantly raised.

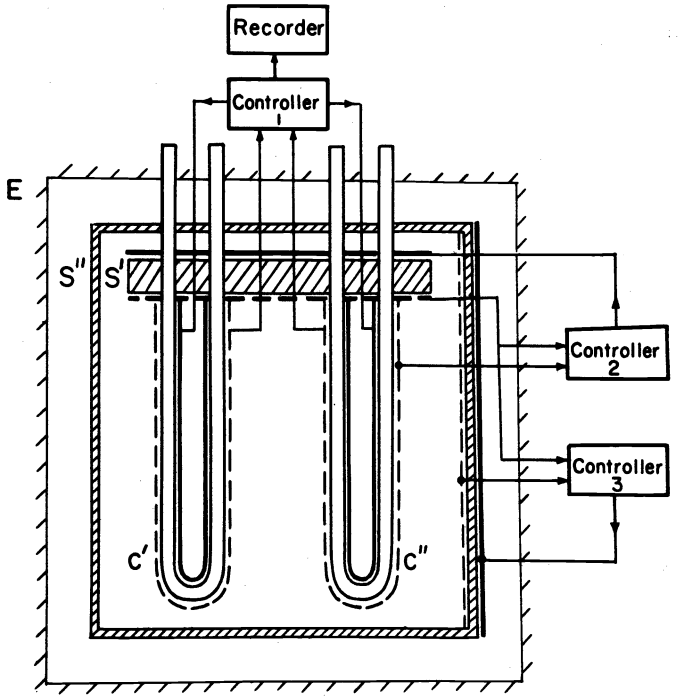

Fig. 9. Scheme of the calorimetric block of the capillary scanning microcalorimeter DASM-1 .

Figure 9 shows the construction of a calorimetric block with capillary cells (Ref. 20), used in the first highly sensitive microcalorimeter DASM-1 manufactured by the Special Bureau of Biological Instrumentation of the Academy of Sciences of the USSR in 1972. In this construction an electric heating element and thermoresistor are uniformly distributed along the surface of the capillary. The capillary cells have been used also in the latest DASM-4 model designed in 1978. Here the capillary cells are folded into a compact helix and are enclosed into a "sombrero" type double adiabatic shell (Fig. 10). Between the helical cells is mounted a 200 junction thermopile used as a thermosensor.

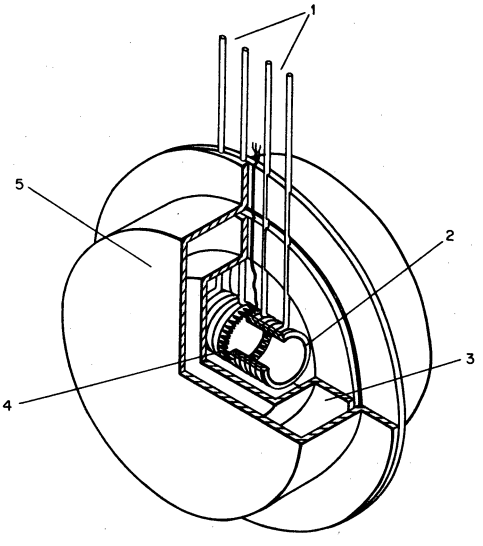

Fig. 10. Calorimetric block of the capillary scanning microcalorimeter DASM-4. 1 - capillary inlets; 2 - capillary cell heater; 3 - internal shield; 4 - thermopile; 5 - external shield.

The calorimetric block of DASM-4 is, in fact, a hybrid of the cells of calorimeters DASM-1 (Fig. 9) and DASM-1M (Fig. 8).

MAIN CHARACTERISTICS OF SCANNING MICROCALORIMETERS

Sensitivity, precision and accuracy

Usually the sensitivity of an instrument implies the minimum signal which can be detected against the noise background. Therefore it depends not only on the noise of the instrument but also on the shape of the signal.

In the case of scanning microcalorimetry of macromolecules the spectrum of possible signals varies within a wide range - from fractions of a degree (melting of homopolymers and phospholipid bilayers) to dozens of degrees (gradual changes of heat capacity, cooperative transitions with small enthalpies) - and the problem of their isolation against the noise background requires detailed studies of noise characteristics, i.e. the noise spectrum of the instrument. However, this is usually somewhat arbitrarily substituted by testing the possibility of isolation of a rectangular power signal with a width of several degrees. As for the noise per se, its evaluation is reduced to an evaluation of a mean square deviation of the recording from an ideal line which could have been obtained if there had been no noise:

$$\delta\overline{W} = \left(\sum_i (\delta Y_i)^2 \, \delta T_i / \Sigma \delta T_k \right)^{1/2} \qquad (6)$$

where δY_i is the minimum deviation of the recording in power units in the range of temperatures δT_i. Here the variable is temperature and not time, since a scanning microcalorimeter usually makes recordings on a temperature scale and not on a time scale.

The most complicated point in evaluation of the mean deviation of the recording is the problem of the zero line corresponding to the ideal recording without noise. It would not be difficult to obtain such a line if it were possible to reproduce the recording repeatedly and to average the obtained results. Unfortunately, in scanning microcalorimetry this is not always possible. At a repeated recording the line can be shifted and its general slope and approximate shape can be changed. Therefore an ideal zero line can be drawn only on a relatively small region of recording when it is more or less proper to consider it as a straight one, or it can be obtained as a smooth line using a smoothing procedure of the following type:

$$\overline{y(\overline{T})} = 1/\Delta T \int_{T-1/2\ \Delta T}^{T+1/2\ \Delta T} y(T)\,dT. \qquad (7)$$

In this case, the greater the interval ΔT, the more temperature-elongated (and consequently, time-elongated) deviations will be smoothed out. Using different intervals ΔT and counting deviations from the corresponding smoothed lines, we could thus obtain a frequency characteristics of the noise. But usually only an interval of several degrees is used for smoothing. In this case the deviation determined by eq. (6) will characterize the intensity of rather midfrequency noise or the sensitivity of the instrument to relatively sharp processes. To evaluate the capability of instrument, in measuring processes extended in temperature it is necessary to evaluate the level of low-frequency (or even extremely low-frequency) noise with the period corresponding to the overall time of heating. This can be done only by averaging the deviations at repeated recordings without refilling the cells of the calorimeter. The line obtained by averaging many recordings of the same sample corresponds to the real zero line of the instrument. The average deviation from this line determines an averaged sensitivity of the instrument to signals whose length varies from fractions of a degree up to the whole temperature range of the instrument. This value, which is usually called the reproducibility of the base line without refilling the cells, determines the precision of a scanning microcalorimeter.

The next step in characterizing a scanning microcalorimeter is its accuracy. It depends not only on the accuracy of determination of the temperature dependence of heat capacity, but on the accuracy with which the instrument can determine the specific or partial heat capacity in all the working range of temperatures.

The relative error of determining the specific heat capacity with a scanning microcalorimeter includes several components:

$$\delta C_p/C_p = \delta W/W + \delta v/v + \delta p/p + \delta m/m \qquad (8)$$

where $\delta W/W$ is a relative error in estimating the position of the base line which is determined by the reproducibility of the recording obtained for empty cells; $\delta v/v$ is a relative error in determining the heating rate; $\delta p/p$ is a relative error in calibrating the instrument; $\delta m/m$ is a relative error in determining the amount of the sample.

A scanning microcalorimeter is usually calibrated electrically by supplying an additional calibration power into one of the cells. The error of electrical calibration can be easily reduced to 0.1% using instruments of a corresponding class. A relative error in determining the rate is of the same order of magnitude. However, a relative error in determining the amount of the sample at small amounts used in microcalorimetry increases to a value of about 1%.

As for the error of $\delta W/W$, it greatly depends on the power, W, necessary for heating at a given rate, i.e. on the heating rate since δW does not significantly depend on the heating rate. Thus, it is advantageous to measure heat capacity at a maximally possible heating rate. But, in any case, the relative error in determining specific heat capacity with a scanning microcalorimeter is no less than 1% due to the error in determining the amount of the sample. It is clear that at such an accuracy of measuring the specific heat capacity, we cannot determine the difference heat capacity of the solution and the solvent even for a 1% solution. The difference in heat capacities for 1% water solutions of macromolecules and water is about 0.7% of the heat capacity of the solution.

In the case of microcalorimeters with a completely filled measuring volume we have for the relative error of the difference heat capacity:

$$\delta \Delta C_p/\Delta C_p = \delta \Delta W/\Delta W + \delta v/v + \delta p/p + \delta V/V + \delta \Delta \rho/\rho \ . \qquad (9)$$

Here instead of the relative error in determining the amount of the sample we have the error in determining cell volume V and the difference of the densities $\Delta\rho$ of the solution and the solvent. However, the error in determining the cell volume is a systematic one and can be essentially reduced by calibrating the instrument with standard samples of known heat capacities. The error in determining the difference in densities is independent of the precision of a scanning microcalorimeter, but depends on the precision of the densimeter or the accuracy of calculation. Therefore, in considering the precision of a scanning instrument these errors can be neglected. (It should be noted only that the precision of modern densimeters allows to make it less than 1%). Since the error of calibrating and determining the rate is here the same as in the above case, it appears that the precision of a scanning microcalorimeter in measuring the difference heat capacity is determined mainly by the $\delta\Delta W/\Delta W$ component, i.e. by the relative error in determining the distance between the recordings obtained for the standard sample (the solvent) and the sample studied (the solution). Here $\delta\Delta W$ is the mean deviation of the recording obtained at repeated measurements with a refilling of the cells, ΔW is the difference of powers applied to the cells with the standard sample and the sample studied to heat them with the same rate. The latter value depends on the difference of heat capacities of the standard and the sample (and consequently, on the concentration if we are dealing with solution) and also on the heating rate. A rise in the heating rate results in an increase of the ΔW value. At the same time, a rise in the heating rate gives also an increase of the $\delta\Delta W$ which is determined by the irreproducibility of heat capacity of the cells at refilling rather than by the noise of the measuring device. Thus, the $\delta\Delta W/\Delta W$ value is practically independent of the heating rate, and since the relative error in estimating the differential heat capacity is determined mainly by this term, is does not significantly depend on the heating rate either. Hence we obtain for the error in determining the difference heat capacity:

$$\delta \Delta C_p \simeq \delta \Delta W/\Delta W \times \Delta C_p = \delta \Delta W/v \qquad (10)$$

where v is the heating rate. The value of $\delta\Delta W/v$ is an average deviation of recordings at repeated measurements with a refilling of the cells, estimated not in power but in heat capacity units. Thus, the accuracy of evaluation of the difference heat capacity with a scanning microcalorimeter is determined by the mean deviation of recordings (in heat capacity units) obtained upon refilled cells. As a rule, such an evaluation is done for cells not filled with the sample studied but with the standard sample and therefore it is called the reproducibility of the base line at refilling. It follows that its value can be regarded as a parameter characterizing the accuracy of the instrument.

In contrast to accuracy, it is worthwhile to express the sensitivity and precision of a microcalorimeter not in heat capacity units but in power units. This is connected with the fact that the value of the average noise level estimated by the recordings at empty cells reflects mainly the disturbances caused by the operation of the electronic system of the calorimeter which measures the difference between powers and not between heat capacities. If we express the noise level and the sensitivity of the calorimeter in heat capacity units, it will depend on the heating rate and will be inconvenient for characterizing the instrument.

Reduced characteristics of a scanning microcalorimeter

It is evident that the sensitivity of a scanning microcalorimeter depends directly on the amount of the sample studied. The less the amount of the sample and, consequently, its heat capacity, the smaller heat effects are registered at heating. Therefore one of the widespread methods of increasing the apparent sensitivity of a scanning microcalorimeter is a reduction of the volume of calorimetric cells. However, in studying the solutions, it is the sensitivity to the heat effect occurring within the sample of a definite volume that is significant and not the absolute sensitivity to the heat effect. For studying solutions minimal concentrations and not minimal volumes are of importance. We shall denote the characteristics of a scanning microcalorimeter, reduced to a unit of volume, as reduced characteristics. It is clear that the reduced characteristics are the basic ones in estimation of the quality of the instrument designed for studying solutions.

Characteristics of some commercially available instruments

Although at present many companies manufacture scanning calorimeters, it is difficult to have a clear idea on the possibilities of these instruments from prospectus since usually the description gives only one or two parameters which are considered to be the most advantageous, for example, only the noise level.

TABLE 1. Main characteristics of commercial scanning microcalorimeters

Quality	Characteristic	Unit	Perkin-Elmer DSC-2	Dupont 910 DSC	Daini Seikoshi SSC-50	Acad.Sci. USSR DASM-1M	Acad.Sci. USSR DASM-4
Operational range	Volume of the cell	ml	0.03	0.03	0.07	1.0	0.5
	Temperature range	K	100-1000	100-1000	120-400	273-373	250-400
	Heating rates	K/min	0.3-320	0.5-100	0.01-5.0	0.1-2.0	0.1-4.0
Sensitivity	Noise level	μW	17	4	1.3	0.5	0.2
	Reduced noise level	μW/ml	600	150	20	0.5	0.4
Precision	Reproducibility without refilling of cell	μW	-	20	2.5	2.0	0.5
	Reduced reproducibility without refilling of cell	μW/ml	-	700	30	2.0	1.0
Accuracy	Reproducibility at refilling of cell	mJK^{-1}	-	-	-	0.3	0.05
	Reduced reproducibility at refilling of cell	mJK^{-1}/ml	-	-	-	0.3	0.1
	Relative error in the heat capacity determination	%	1.0	1.0	0.5	0.01	0.005

The Table summarizes the basic characteristics which we have derived from the descriptions and illustrations of some manufactured instruments which at present are most frequently used in studying macromolecules. As seen, though there is only a 100-fold difference in the noise level of the considered instruments, their reduced noise level which per se determines their fitness for studying macromolecules in solution, differs by more than 1000, while their relative error in estimating heat capacity varies by two orders of magnitude. This qualitative difference compels us to divide scanning calorimeters into two classes. The dividing line can be the relative error in estimating heat capacity. It is expedient to consider the instruments for which this error does not exceed 0.1% as precision ones, as these instruments can be used in quantitative studies and, in particular, studies of macromolecules in solution. As for the other instruments, which we cannot call precision ones, the Table shows that they have a number of various advantages: a smaller volume of the cell, larger ranges of heating rates and working temperatures. In other words, they are more universal. It is even likely that precision is the cost for universality, which has ensured their wide usage mainly in applied studies. Such instruments are also used in studying macromolecules in solution: low sensitivity and accuracy is compensated by using high concentrations and heating rates, but possibilities of their increase are rather limited. The maximally admissible heating rate in studying macromolecules is likely to be the rate of 5 K/min, but in many cases it is much lower.

INFORMATION OBTAINED BY SCANNING MICROCALORIMETRY

Processing of the calorimetric data

Most of the existing scanning microcalorimeters determine the temperature dependence of the heat capacity and not of enthalpies. Though enthalpy and heat capacity are related functions and can be easily determined one from another, the direct measurement of heat capacity is preferable, since heat capacity as a derivative of enthalpy describes more precisely its change with temperature.

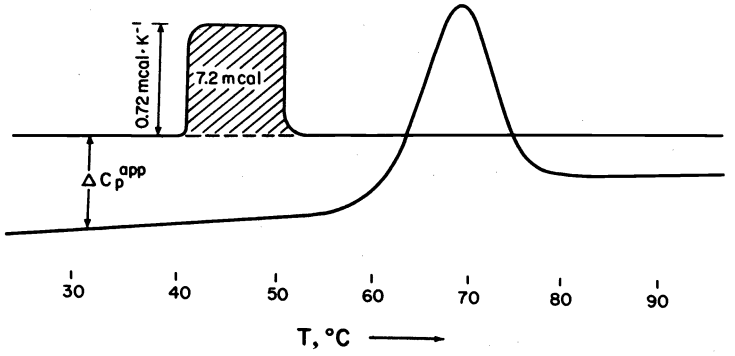

Fig. 11. Microcalorimetric recording of the temperature dependence of heat capacity of the lysozyme solution obtained on the scanning microcalorimeter DASM-1M. (Solution concentration 0.152%, pH 2.5, heating rate 1 K/min).

Figure 11 represents a typical microcalorimetric recording obtained for relatively simple macromolecular systems (a globular protein lysozyme) in which drastic changes of a state (denaturation) occur in a certain temperature interval. The horizontal line in the figure represents the base line, i.e. the recording obtained when both cells of the calorimeter are filled with the solvent. The hatched square on the base line is the calibration mark obtained by the supply of definite power into one of the cells. As seen, the heat capacity of the lysozyme solution in the whole range of temperatures (except that of the denaturational heat absorption peak) is lower than the heat capacity of the same volume solvent. This is explained by the fact that the partial heat capacity of a protein in solution is lower than that of the same volume solvent (21). The observed difference in heat capacities $\Delta C_p^{app}(T)$ can be represented as

$$-\Delta C_p^{app}(T) = C_{p,pr}(T)\, m_{pr}(T) - C_{p,s}(T)\Delta m_s(T) \qquad (11)$$

where $C_{p,pr}(T)$ is the protein partial heat capacity, $m_{pr}(T)$ is the protein mass in the cell at temperature T, $C_{p,s}(T)$ is the specific heat capacity of the solvent and $\Delta m_s(T)$ is the solvent mass displaced by the protein at temperature T and is equal to

$$\Delta m_s(T) = m_{pr}(T)\ V_{pr}(T)/V_s(T)\ . \qquad (12)$$

Here $V_{pr}(T)$ is the protein partial specific volume and $V_s(T)$ is the solvent specific volume at temperature T.

From (11) we obtain for the protein partial heat capacity in solution:

$$C_{p,pr}(T) = C_{p,s}(T)\ V_{pr}(T)/V_s(T) - \Delta C_{pr}^{app}(T)/m_{pr}(T)\ . \qquad (13)$$

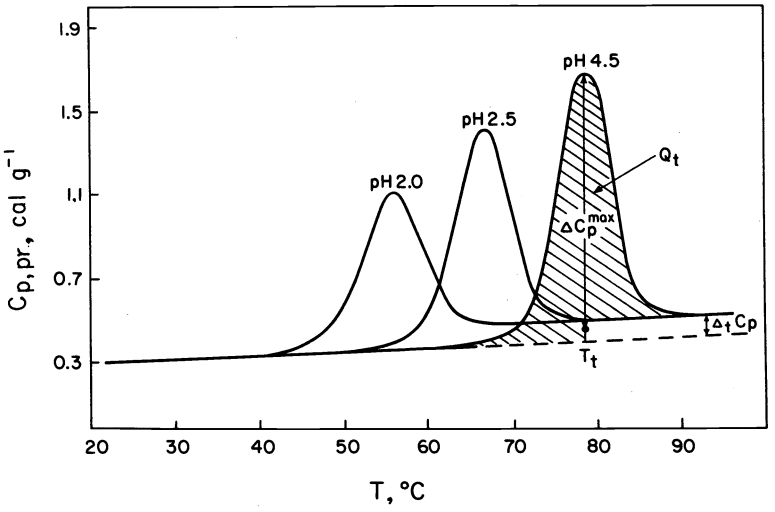

Fig. 12. Temperature dependence of the lysozyme partial heat capacity in solutions with different pH.

Figure 12 represents temperature dependences of the lysozyme partial heat capacity in solutions with different pH values. It is seen that the initial partial heat capacities coincide in all cases at 20°C and then change similarly with temperature up to the denaturational temperatures which are determined by the pH value of the given solution. After denaturation the partial heat capacities are equal again. The question arises: how would one determine the enthalpy of the denaturation process?

To do this, usually heat capacities at the beginning and at the end of the process are connected by the straight line and the area of the peak above this line is considered as enthalpy of transition from one state (native) to another (denatured). However, it is clear that such a simplified procedure is not quite correct when heat capacities of the initial and final states are not equal. In fact, to determine enthalpy of the temperature-induced process, we must evaluate the contribution of the "trivial" heat capacity of the initial and final states in the observed heat effect. This can be done more or less easily only in the case of a transition between two states when the relative concentration of states can be determined within the transition zone. In this case the "trivial" heat capacity is presented by a sigmoidal curve connecting heat capacity values corresponding to the initial and final states. But usually the sigmoidal curve is substituted by the stepwise curve which is obtained by linear extrapolation of heat capacities of the initial and the final states to the middle of transition T_t. Since $\Delta_t C_p$ determines temperature dependence of enthalpy, for enthalpy function we have:

$$\Delta H(T) = \Delta_t H + \int_T^{T_t} \Delta_t C_p dT\ . \qquad (14)$$

It can be seen that it just corresponds to the area of the heat capacity peak with a step at temperature T.

For the entropy function we have accordingly:

$$\Delta S(T) = \Delta_t H/T_t + \int_{T_t}^{T} \Delta_t C_p \, d\ln T . \qquad (15)$$

It is much more difficult to determine enthalpy and entropy when the process is a complex one and consists of several sequential stages as shown in Fig. 13.

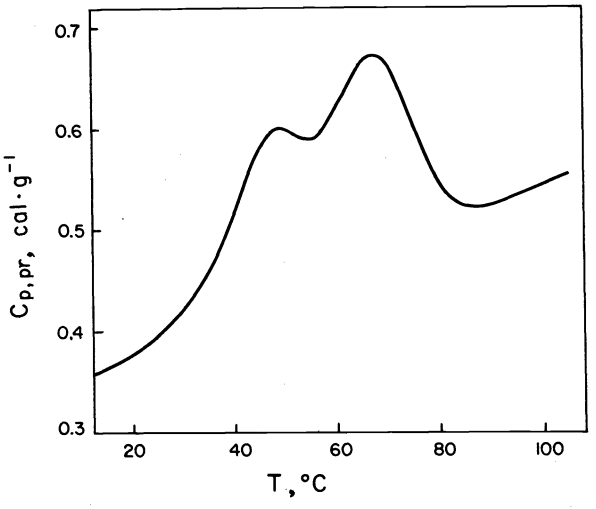

Fig. 13. Temperature dependence of partial heat capacity of the TN-C (calcium-binding component of troponin) in a calcium buffer (10 mM KOH, pH 7.2, 10 mM EDTA and 9 mM $CaCl_2$).

In this case we have to assume that (a) either the change of the system heat capacity proceeds similarly at each stage; (b) or the heat capacity change at different stages is proportional to the heat absorbed at this stage; (c) or the heat capacity change mainly proceeds at one of the stages. A choice between these possibilities can be based only on indirect evidence and model considerations.

Analysis of the shape of the heat absorption curve

As has been mentioned in the previous section, the quantitative processing of the curve describing the temperature dependence of the experimentally observed heat absorption requires information on the process itself: whether the process is simple or complex, i.e. whether it represents a transition between two macroscopic states or is a set of transitions between several states. It is, however, clear that in most cases this question in itself represents a problem and its elucidation is necessary not only to process the curves, but to understand the studied macromolecular systems. One of the most remarkable features of the calorimetric curves is that such information can be obtained directly from the analysis of the shape of these curves without using any additional data. In other words, the calorimetric curves of the heat capacity temperature dependence, in contrast to any other temperature dependence, contain all the information necessary for a complete thermodynamic description of the system and it can be obtained by analysis of the shape of these curves. This is due to the fact that the enthalpy of the process which can be represented as

$$\langle \Delta H \rangle = \sum_{i-1}^{n} \Delta H_i \exp(-\Delta G_i/RT)/Q \qquad (16)$$

where ΔH_i and ΔG_i are enthalpy and Gibbs energy of the i-th transition and Q is the partition function determined not by 3(n-1) independent parameters as for any other function describing the process, but by 2(n-1) parameters. This decrease of the number of parameters is already sufficient to determine all of them from one curve (Ref. 22). However, it should be kept in mind that though this problem can, in principle, be solved, its practical realization is possible if (a) the observed process presents a reversible equilibrium; (b) the precision of the curve plotting is sufficient to obtain all the necessary parameters. From the general considerations it is clear that with the complication of the process the requirements for the precision and reliability

of the experimental curve will increase and its quantitative analysis will become more complicated.

Let us begin the consideration of this problem from the simplest case when only one peak of a simple shape is observed on the heat capacity temperature function.

In this case assuming that the macromolecule has only two thermodynamically stable states, we can express the equilibrium constant through any parameter sensitive to the state $K=\upsilon/1-\upsilon$. Since the change of K with temperature is determined by the van't Hoff equation:

$$d \ln K/d\cdot 1/T = d\ln(\upsilon/1-\upsilon)/d(1/T) = \Delta H.$$

For enthalpy of the process we have:

$$\Delta H = RT^2/(1-\upsilon)\ \upsilon\ d\upsilon/dT \qquad (17)$$

and for the temperature T_t corresponding to the transition mid-point

$$\Delta_t H = 4RT_t^2 \quad (d\upsilon/dT)_t \ . \qquad (18)$$

However, for the calorimetric curve the parameter υ, determining the progress of the reaction, is the ratio of the heat absorbed at the given temperature Q(T) to the total heat Q_t

$$\upsilon(T) = Q(T)/Q_t \ .$$

Thus,

$$\Delta H = 4RT_t^2/Q_t \ (dQ/dT)_t = 4RT_t^2\ \Delta C_p^t/Q_t \ . \qquad (19)$$

Here ΔC_p^t is the height of the heat absorption peak at the middle of the transition; Q_t is the peak area. This enthalpy of the transition is usually called the van't Hoff enthalpy and it is designated by the corresponding superscript ΔH^{VH}. If the considered process really represents a two-state transition, the van't Hoff enthalpy must be equal to the real one determined calorimetrically from the heat absorption peak area:

$$\Delta H^{cal} = MQ_t \ . \qquad (20)$$

In other words, the ratio

$$\Delta_t H^{cal}/\Delta_t H^{VH} = MQ_t/4RT_t^2\Delta C_p^t \qquad (21)$$

must be close to one. Thus, the answer to the question whether the observed process is a transition between two states boils down to a simple analysis of the shape of the peak, to the determination of the ratio of its area to its height. For the peaks in Fig. 12 representing the lysozyme denaturation, the ratio

$$\Delta H^{cal}/\Delta H^{VH}$$

is very close to one. This ratio is very close to one for many small compact globular proteins (Ref. 21). It follows that denaturation of these proteins can be considered in a first approximation as a simple transition between two discrete macroscopic states. At the same time, if we take such a globular protein as papain, it turns out that though the peak of its denaturational heat absorption differs little in appearance from that observed for lysozyme (see Fig. 14), the ratio

$$\Delta H^{cal}/\Delta H^{VH}$$

for this process is close to two (Ref. 24). Hence it can be concluded that melting of this protein includes two independent transitions or that there are two independent cooperative regions in its structure. In fact, the papain globule has a deep cleft in the middle which divides its structure into two domains. Quite an opposite example is given by another globular protein, pancreatic trypsin inhibitor, for which

$$\Delta H^{cal}/\Delta H^{VH} = 0.5 \ .$$

Such a deviation of enthalpies can be explained only by the assumption that the cooperative unit of the pancreatic trypsin inhibitor at elevated temperature is not a monomer molecule, but at least a dimer.

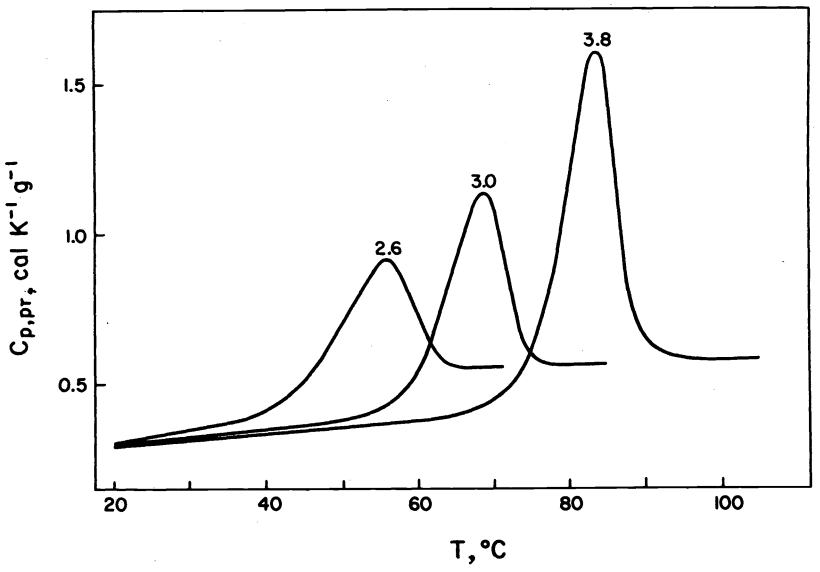

Fig. 14. Temperature dependence of papain partial heat capacity.

An even more striking example is presented by the phospholipid system in aqueous medium. Figure 15 represents heat absorption observed at the heating of phospholipid dipalmitoyl lecithin. It is seen that this process is complex: there are two peaks, but they are clearly apart and our analysis is applicable

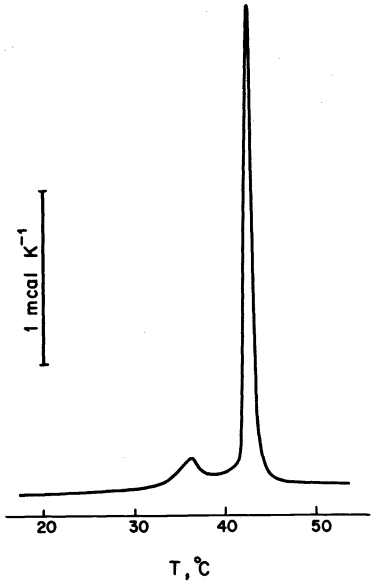

Fig. 15. Heat absorption at melting of dipalmitoyl lecithin liposomes in water recorded on the instrument DASM-1M. Concentration of solution 0.027%.

to each of them. This analysis shows that $\Delta H^{cal}/\Delta H^{VH}$ is of the order of 0.01 for both peaks. Hence we can conclude that not a phospholipid molecule, but a whole cluster containing about a hundred molecules is a cooperative unit in this system (see Ref. 14). Such an analysis becomes much more complicated when there are many peaks which partially overlap as in the case of melting of transfer RNA molecules (Fig. 16). Analysis can be more or less easily done when the complex process represents an additive sum of n simple processes, i.e. the constituent elementary transitions are independent and their enthalpies do not depend on temperature. In this case:

$$\Delta H = \sum_{i=1}^{n} \Delta H_i \qquad (22)$$

$$\Delta H_i = \Delta H_i^{VH} \qquad (23)$$

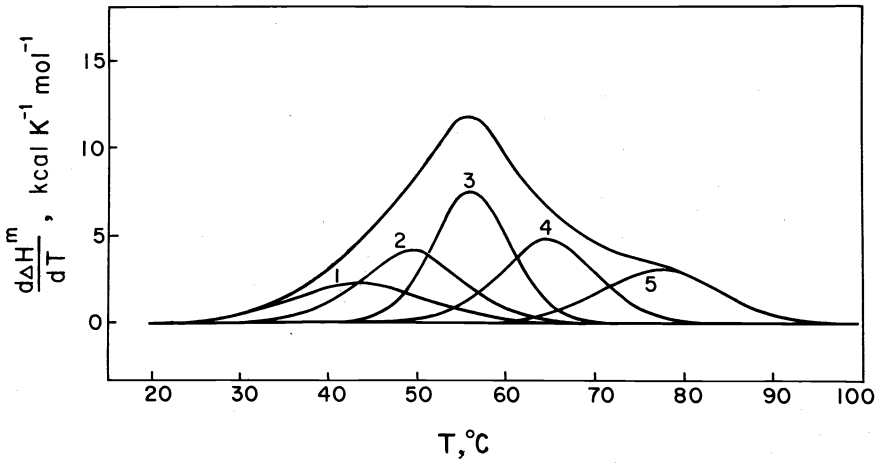

Fig. 16. Heat absorption at melting of the transfer RNA^{Phe} structure per mole of molecule.

or taking into account (19)

$$\Delta H_i = 2R^{1/2} T_i^{max} (\Delta C_p^{max})^{1/2}. \quad (24)$$

Equations (22) and (24) strictly determine the shape of the elementary peaks into which the observed heat absorption can be decomposed (for more detail see Ref. 25).

The main advantage of the described approach to the analysis of complicated curves is its simplicity. However, in the case when the elementary processes constituting the complex one are interdependent, this approach cannot be applied, and more detailed analysis of the shape of the observed heat absorption function based on statistical thermodynamics is necessary. Such an approach to the analysis of complicated calorimetric curves has been recently proposed by Freire & Biltonen (23). It is based on the fact that the partition function Q of any system is determined by the enthalpy:

$$Q = \exp \int_{T_o}^{T} \Delta H/RT^2 \, dT \quad (25)$$

where T_o is the temperature at which the system is in the initial state. Since $1/Q=F_o$ is the fraction of molecules in the initial state, for the fraction of molecules in all other states we have:

$$1 - F_o = \exp(-\int_{T_o}^{T} \Delta H/RT^2 \, dT). \quad (26)$$

It is clear that if this value is determined at the very beginning of the observed heat absorption, i.e. close to T_o, it will basically describe the transition from the initial to the first state. Hence

$$\lim_{T \to T_o} \Delta H(T)/1-F_o = \Delta H_1$$

where ΔH_1 is the enthalpy of the first transition. Having subtracted the enthalpy of the first transition from $\Delta H(T)/1-F_o$, we obtain the excess enthalpy $\Delta H_1(T_1)$ corresponding to the enthalpy of all but the first transition. By repeating this procedure several times we obtain enthalpies of all other transitions. Thus, this procedure of deconvolution of a complex process into constituents is based on a sequential analysis of the very beginning of the process and of its fragments where the main contribution is made by only one transition. This situation creates many difficulties, since the weak heat effect at the beginning of the process is not easy to isolate from the background of noise, especially of the low-frequency noise. At the same time, the procedure as it was primarily suggested (see also Ref. 25) leads to accumulation of errors made at subsequent steps. In other words, the error of analysis rapidly increases with the number of transitions constituting the whole process. Therefore, this type of analysis requires highly accurate experimental data, i.e. the high precision and accuracy of the instrument used in experiments.

CONCLUSIONS

The first question one asks about scanning calorimetry is about its sensitivity, precision and accuracy. Are they high enough for quantitative studies of macromolecules?

Though modern precise instruments already enable us to carry out quantitative studies of macromolecules, nevertheless a 5-10-fold improvement of the characteristics achieved is still desirable. This would allow one (a) to work on the microcalorimeter with solutions of the concentrations used for optical studies (0.01%); (b) to spend not milligrams, but fractions of a milligram of a substance which is often decisive in work with biological objects; (c) to carry out a much more detailed quantitative analysis of the data obtained than it is possible now.

The desire to increase sensitivity, precision and accuracy of scanning microcalorimeters is quite natural but the question is whether this is feasible. It seems that in any case a five-fold increase of sensitivity and precision over the latest scanning microcalorimeter DASM-4 is quite possible even without developing new approaches to the heat capacity measurements.

ACKNOWLEDGEMENTS

The author is very grateful to Professor S. J. Gill for reading the manuscript and helpful discussion.

REFERENCES

1. P. L. Privalov, J. R. Monaselidze, G. M. Mrevlishvili and V. A. Magaldadze, Zh. Eksp. Teor. Fiz. (USSR) 47, 2073-2079 (1964).
2. P. L. Privalov, J. R. Monaselidze, G. M. Mrevlishvili and V. A. Magaldadze, Soviet Physics JEPT Letters 20, 1393-1396 (1965).
3. S. J. Gill and K. Beck, Rev. Sci. Instr. 36, 274-276 (1965).
4. R. Danford, H. Krakauer and J. M. Sturtevant, Rev. Sci. Instr. 38, 484-487 (1967).
5. E. S. Watson, M. J. O'Neil, J. Justin and N. Brenner, Anal. Chem. 36, 1233-1238 (1964).
6. E. S. Watson and M. J. O'Neil, US Patent 3,263, 484 (1966).
7. P. L. Privalov, Abstr. Second Internat. Biophys. Congress, Vienna, 5-9 Sept., No. 92, 117 A2/02 (1966).
8. P. L. Privalov, Proc. 1st Internat. Conf. Calorimetry & Thermodynamics, Warsaw (1969).
9. P. L. Privalov, IV Internat. Biophys. Congress, Moscow, Symposial papers, Poustchino, pp. 204-210 (1972).
10. P. L. Privalov, IV Internat. Conf. Chem. Thermodynamics, Montpellier, France; Pure & Appl. Chem. 47, 293-304 (1976).
11. P. L. Privalov, FEBS Lett. 40S, 141S-153S (1974).
12. J. M. Sturtevant, Proc. 1st Internat. Conf. Calorimetry & Thermodynamics, Warsaw (1969).
13. J. M. Sturtevant, Ann. Rev. Biophys. Bioengin. 3, 35-51 (1974).
14. S. Mabrey and J. M. Sturtevant, Methods in Membrane Biol. (Editor E. D. Korn) vol. 9, pp. 237-274, Plenum Press, N. Y. & London (1978).
15. P. L. Privalov and J. R. Monaselidze, Pribory i Tekhnika Eksperimenta (USSR) 6, 174-178 (1965).
16. H. B. Alberts, Rev. Sci. Instr. 4, 766-774 (1972).
17. P. L. Privalov, V. V. Plotnikov and V. V. Filimonov, J. Chem. Thermod. 7, 41-47 (1975).
18. P. Ross and K. Goldberg, Thermochim. Acta 10, 143-151 (1974).
19. P. L. Privalov, P. S. Makurin, V. V. Plotnikov, V. V. Koryagin, V. S. Polpudnikov and G. G. Stepanyuk, USSR Patent G01h No. 276490 (1970).
20. P. L. Privalov, P. S. Makurin, V. V. Plotnikov, V. V. Koryagin, V. S. Polpudnikov and G. G. Stepanyuk, US Patent G01K No. 3899918 (1970).
21. P. L. Privalov and N. N. Khechinashvili, J. Mol. Biol. 86, 665-684 (1974).
22. R. L. Biltonen and E. Freire, Crit. Rev. Biochem. 5, 85-124 (1978).
23. E. Freire and R. Biltonen, Biopolymers 17, 463-479 (1978).
24. E. I. Tiktopulo and P. L. Privalov, FEBS Lett. 91, 57-58 (1978).
25. P. L. Privalov and V. V. Filimonov, J. Mol. Biol. 122, 447-464 (1978).