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NEW ISOTHERMAL TITRATION CALORIMETER FOR INVESTIGATIONS ON VERY SMALL SAMPLES
Theoretical and experimental studies

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Abstract

A new isothermal titration calorimeter with 78.5 μL volume capillary chambers has been developed. It is based on the theory of mixing reagents in a capillary chamber of a titration calorimeter and separation of the sensitive volume of chambers in a differential calorimeter. The evaluation of the efficiency of diffusion mixing is described by means of an oscillating dispensing needle. The calorimeter was tested by the reactions: Ba^{2+} – 18-Crown-6 and Rnase-2'CMP. The main advantages of the new titration calorimeter are the use of very small amounts of reagents, the high accuracy of separating the sensitive volume of calorimetric chambers and the minimization of power input while mixing reagents in a horizontally located capillary chamber.

Keywords: capillary calorimetric chamber, differential titration calorimeter, mixing reagents in a capillary chamber, sensitive volume of calorimetric chambers

Introduction

Titration calorimetry is widely used as a method for thermodynamic description of a chemical reaction. A new isothermal titration calorimeter is designed to measure fine heat effects of interaction between biological molecules (proteins and peptides, membrane systems, nucleic acids, low-molecular compounds and other objects) [1].

Titration calorimeters have been actively developed since the early 1960s [2–4]. One of the first calorimeters is described in [5]. This calorimeter was made according to the differential principle which later has become basic. Thermostatic control of the calorimetric unit is done by a thermostat maintaining temperature with an error of 0.02°C. The instrument was foreseen to measure heats of the order 0.001 cal for a calorimetric chamber volume of about 5 mL. The tendencies laid in this instrument have been well preserved in the titration calorimeters of next generations.

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Tendencies in building titration calorimeters

Freire [6] made an analysis of the main solutions on which the calorimeters of the 1990s having a stirrer are based. It was shown that the stirrer gave rise to a continuous heat effect which revealed in a base line drift. If the mechanical stirrer was inadequately aligned, the noise level grew which restricted the effective sensitivity of the instrument. The presence of a stirring blade in the solution limited the possibility of miniaturization of the chambers. Since the stirrer was in contact with the reagents and the internal environment, it became a heat conducting way affecting the experiment at temperatures different of ambient temperature. The stirrer displaced some reagent amount from the reaction chamber, and its volume should be known with high precision to be reproduced from one experiment to another. This could be complemented by the fact that in titration calorimeters implementing the known principles of thermostatic control of adiabatic scanning calorimeters, reliable isothermal mode is not provided.

Another version of titration calorimeters of the 1990s are calorimeters based on the hydraulic 'jet' pulse agitation method.

Mixing of reagents in the calorimetric chamber is performed by feeding them as high-speed jet flows [6]. The system of feeding reagents requires equalization of powers fed by these flows into each chamber. The paper presents data on equalization of the power of these flows up to 12 μJ, which is one of the essential problems requiring further studies. The theory of mixing the reagents by the JET method is not described and no sufficient experimental data are reported corroborating the efficiency of the mixing method for different substances or conditions.

Isothermal conditions are provided by a shield, the temperature of which is maintained constant. The excess heat is drawn off from the chambers to the shield. Due to the presence of feeding tubes of the calorimetric chambers, their sensitive volume comprises some length of these tubes, which does not permit any accurate determination of the sensitive volume. Indeed, a part of interaction heat is dispersed directly on the shield from the tubes by-passing measuring thermocouples.

The analysis of the methods and practical technical solutions used in titration calorimeters worldwide has revealed the following tendencies in building titration calorimeters: (1) differential structure, (2) inclination to use minimum amounts of reagents, (3) reliable component concentration equalization in the measuring chamber volume, (4) ensuring high accuracy of measuring, (5) providing strongly isothermal conditions.

While developing the method of titration isothermal calorimetry for physicochemical investigations on very small samples, the above tendencies have been taken into consideration.

To choose technical solutions for obtaining isothermal conditions and separation of the sensitive volume as well as the heat and mass exchange in the calorimetric chambers, the earlier experience in the developing scanning calorimeters was taken into account. A possibility of further development of these solutions as applied to the

requirements of isothermal calorimetry has been studied and corroborated in the new methods described herein.

Mixing reagents in a capillary calorimetric chamber of a titration calorimeter

A capillary calorimetric chamber was chosen for an isothermal titration calorimeter after evaluating the efficiency of mixing a ligand and a sample in such a chamber. The time of equalization of the ligand concentration in the volume of the sample as a result of concentration diffusion was taken as a criterion. For experimental corroboration of the calculated time of equalization, is a model system NADH–LDH the time of binding of which is negligible 10^{-3} s [7].

Theoretical relationship of the time for diffusion equalization of the ligand concentration along the sample contained in the sensitive volume of a cylindrical calorimetric chamber was determined under the following conditions.

$V_d = \pi R_0^2 L$ is the volume of the band (ligand portion), cm^3;

$V_k = \pi R_k^2 L$ is the sensitive volume of the calorimetric chamber;

R_0 is the radius of the band, cm;

R_k is the internal radius of the calorimetric chamber, cm;

$\rho_1(\tau)$ is the current average value of the ligand concentration (density) in the volume V_d;

$\rho_2(\tau)$ is the current average value of the ligand concentration (density) in the volume $V_p=V_k-V_d$;

$\rho_1=\rho_n\big|_{\tau=0}$ is the initial ligand concentration (density) in the band of volume V_d;

$\rho_2=0\big|_{\tau=0}$ is the initial ligand concentration (density) in the volume of the calorimetric chamber outside the band volume $V_d=V_k-V_d$;

$\rho_k\big|_{\tau=\infty}=\rho_1\big|_{\tau=\infty}=\rho_2\big|_{\tau=\infty}$ is the final ligand concentration (density) in the working volume of the calorimetric chamber V_k;

The final concentration (density) ρ_k (Fig. 1) was calculated as follows.

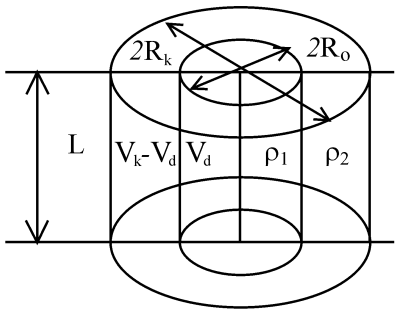

Fig. 1 Fragment of a calorimetric chamber containing a ligand dose

The ligand mass is $m_T=V_d\rho_n=V_k\rho_k$. Hence

$$\rho_k = \frac{V_d\rho_n}{V_k} \tag{1}$$

From the mass conservation law: $\rho_2(\tau)(V_k-V_d)=(\rho_n-\rho_1(\tau))V_d$, which gives

$$\rho_2(\tau) = \frac{(\rho_n-\rho_1(\tau))V_d}{(V_k-V_d)} \tag{2}$$

Specific mass flow from the ligand band surface (at $R=R_0$) having the volume V_d due to the concentration diffusion is determined by Fick's law:

$$q = -D_k \left.\frac{d\rho(\tau)}{dR}\right|_{R=R_0}$$

$$q \approx -D_k \frac{\rho_2(\tau)-\rho_1(\tau)}{R_0} \tag{3}$$

By substituting $\rho_2(\tau)$ from formula (2) in formula (3) we obtain:

$$q = \frac{D_k}{R_0}\left[\rho_1(\tau) - \frac{(\rho_n-\rho_1(\tau))V_d}{V_k-V_d}\right] = \frac{D_k}{R_0(V_k-V_d)}[\rho_1(\tau)V_k - \rho_n V_d] \tag{4}$$

The modification of the ligand mass in volume V_d due to its transport by concentration diffusion can be expressed as:

$$dm(\tau)=m(\tau)-m(\tau+d\tau)=\pi R_0^2 L\rho_1(\tau)-\pi R_0^2 L\rho_1(\tau+d\tau)$$

Since

$$\rho_1(\tau+d\tau)\approx\rho_1(\tau)+\frac{d\rho_1(\tau)}{d\tau}d\tau$$

where τ is the current time, then

$$dm(\tau) = \pi R_0^2 L[\rho_1(\tau)-\rho_1(\tau+d\tau)] = -\pi R_0^2 L\frac{d\rho_1(\tau)}{d\tau}d\tau \tag{5}$$

On the other hand, the mass variation can be determined as $dm(\tau)=qFd\tau$, where $F=2\pi R_0 L$ is the surface area of the band, thus

$$dm(\tau) = \frac{2\pi LD_k}{V_k-V_d}[\rho_1(\tau)V_k-\rho_n V_d]d\tau \tag{6}$$

Setting equal Eqs (5) and (6) for $dm(\tau)$, we obtain:

$$-\frac{R_0^2 d\rho_1(\tau)}{d\tau} = \frac{2D_k}{V_k-V_d}[\rho_1(\tau)V_k-\rho_n V_d]$$

hence

$$\frac{d\rho_1(\tau)}{d\tau} = -\frac{2D_k V_k}{(V_k - V_d)R_0^2}\rho_1(\tau) + \frac{2D_k V_d}{(V_k - V_d)R_0^2}\rho_n \quad (7)$$

which is reduced to a differential equation $dy/d\tau = -py$.

Solving this equation for time τ of equalization of the ligand concentration in the sample contained in the sensitive volume of the calorimetric chamber, we obtain:

$$\tau = \frac{R_0^2}{2D_k}\left(1 - \frac{V_d}{V_k}\right)\ln\left[\frac{1}{k}\left(\frac{V_k}{V_d} - 1\right)\right] \quad (8)$$

where $k = (\rho_1(\tau) - \rho_k)/\rho_k$.

The value of D_k is determined experimentally. For this purpose lactate dehydrogenase [E.C. 1.1.1.27] was isolated from porcine muscle according to a modified Jescai method [8] and additionally purified on DEAE cellulose, the major protein not adsorbed on cellulose was an electrophoretically homogeneous isoenzyme M_4. LDH concentration was determined spectrophotometrically using $E_{280}^{1\%,1cm}$=12.9 in 0.1 M phosphate buffer, pH 7.

Figure 2 represents an adsorption spectrum for an LDH sample of 400 μL measured with a Specord spectrophotometer immediately after injection of a NADH ligand dose of 33 μL. The time (τ_{exp}) of NADH concentration equalizing in a LDH solution was 412 s. The fact of completing the process of NADH concentration equalization in a LDH solution was confirmed by the invariability of the stationary optical density value after vigorous agitation of the optical cell content. The obtained D_k

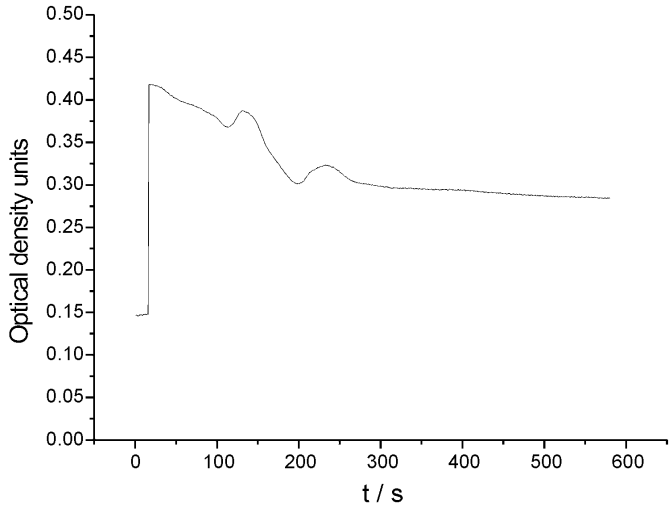

Fig. 2 Measured curve of optical absorption of NADH in LDH
LDH sample 4.28 μM 400 μL, NADH titrant 0.5 mM, 33 μL. Measurements were performed on a Specord M40 Spectrophotometer at 340 nm. Time of NADH concentration equalization in the cell volume is 412 s

value is $1.75 \cdot 10^{-5}$ cm^2 s^{-1} with the error of ligand concentration equalization being 5%. The theoretical value of equalization time τ of 37 s was determined for our cylindrical capillary calorimetric chambers (Eq. (8)) using the obtained D_k. The calculations of D_k and τ values are given in Appendix.

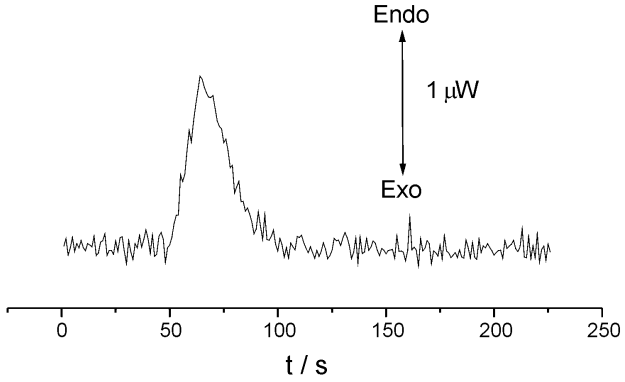

Fig. 3 Measured curve of heat power for NADH interaction with LDH in the KTD calorimeter. LDH sample 4.28 μM, NADH titrant 0.5 mM, 3.5 μL. The time of the recorded heat effect signal is 40 s

The time of the thermal effect of interaction between NADH and LDH (Fig. 3) determined experimentally is 40 s. Thus, the time of the thermal effect of interaction between NADH and LDH practically corresponds to the calculated time of diffusion concentration equalization in the volume of a cylindrical capillary calorimetric chamber.

Separating the sensitive volume of calorimetric chambers in a titration calorimeter

The conception of a technical solution for separating the sensitive volume of calorimetric chambers in a calorimeter and for realizing a heat-conducting bridge is described in detail in our paper [9]. Here we give only a short account of this paper necessary to understand the method of isothermal titration calorimetry for physicochemical studies of very small samples.

Figure 4 presents a heat model of a calorimetric chamber. The sensitive volume of the chamber is represented by a part of a capillary tube located below the heat-conducting bridge 1. It is shown that there exist conditions when the part of the heat measured by the calorimeter and resulting from heat processes occurring in the portion of the capillary tube situated above the lowest limit of the heat-conducting brid- ge 1, is equal to zero. This phenomenon is explained as follows: the recorded heat process is determined as:

$$Q_{rec} = Q_{sv} + KQ_{br} = Q_{sp}\rho_{liq}(V_c + KV_{br}) \quad (9)$$

where Q_{sv} is the heat effect released in the sensitive volume of the calorimetric chamber; Q_{br} is the heat effect released in the volume of the heat-conducting bridge V_{br} which contacts the sensitive volume V_c; Q_{sp} is the specific heat effect of the reaction, ρ_{liq} is the density of the liquid, K is the factor influence the thermal effect in the volume V_{br} on the thermal effect registered in the sensitive volume of the calorimetric chamber V_c.

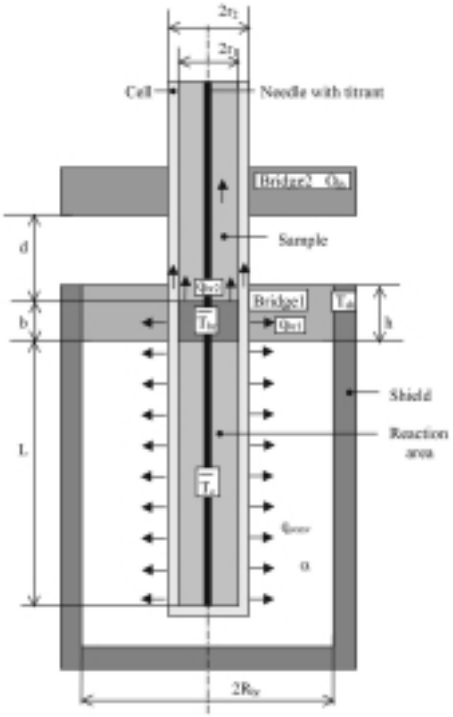

Fig. 4 Schematic drawing of the calorimetric unit with heat bridges 1 and 2

The error of measuring the thermal effect in the sensitive volume of the calorimetric chamber V_c depending on the influence of the thermal effect in volume V_{br} may be determined as:

$$\delta = \frac{Q_{rec} - Q_{sv}}{Q\text{sv}} 100\% = K \frac{b}{L} 100\% \quad (0 \le b \le h) \tag{10}$$

The factor of influence K can be expressed as ratio of the heat flow coming into the sensitive volume of the calorimetric chamber and recorded by the calorimeter to the sum of the heat flows non-recorded by the calorimeter:

$$K = \frac{q_{conv}}{q_{br_1} + q_{br_2}} \tag{11}$$

The heat flow carried away by convection from the sensitive volume of the calorimetric chamber:

$$q_{\text{conv}} = 2\pi r_2 L\alpha(\overline{T}_{\text{c}} - T_{\text{sh}}) \tag{12}$$

The heat flow carried away from the volume V_{br} through the cylindrical bridge 1 to the shield:

$$q_{\text{br}_1} = \frac{2\pi\lambda_{\text{br}} h(\overline{T}_{\text{br}} - T_{\text{sh}})}{\ln\dfrac{R_{\text{br}}}{r_2}} \tag{13}$$

The heat flow carried away from the volume V_{br} to the heat-conducting bridge 2:

$$q_{\text{br}_2} = \frac{\pi(\overline{T}_{\text{br}} - T_{\text{sh}})}{d}[(r_2^2 - r_1^2)\lambda_{\text{c}} + r_1^2\lambda_{\text{liq}}] \tag{14}$$

Using the above equations we obtain the final K value:

$$K \approx \left[\frac{2\alpha L r_2}{\dfrac{2\lambda_{\text{br}} h}{\ln\dfrac{R_{\text{br}}}{r_2}} + \dfrac{(r_2^2 - r_1^2)\lambda_{\text{c}} + r_1^2\lambda_{\text{liq}}}{d}}\right] \cdot \left[\frac{1}{1 + \dfrac{\alpha L^2 r_2}{(r_2^2 - r_1^2)\lambda_{\text{c}} + r_1^2\lambda_{\text{liq}}}}\right] \tag{15}$$

where the heat transfer factor $\alpha \approx 2\cdot10^{-5}$ W mm^{-2} K^{-1}; L=50 mm; h=8 mm; R_{br}=20 mm; r_2=0.6 mm; r_1=0.5 mm; thermal diffusivity of the chamber material λ_{c}=0.07 W mm^{-1} K^{-1} (platinum); thermal diffusivity of the heat-conducting bridge λ_{br}=0.39 W mm^{-1} K^{-1} (copper); thermal diffusivity of liquid λ_{liq}=0.65 W mm^{-1} K^{-1} (water).

For the geometry of the calorimetric chambers and the bridge with L=50 mm and h=8 mm, the value of K=2·10^{-4} and the error of measuring the heat effect is about 0.003%.

Structure of the calorimeter

Based on the new methods developed, a differential titration calorimeter, model KTD-2156, was built (Fig. 5). The concepts of the main technical solutions implemented in the design of the new titration calorimeter with capillary calorimetric chambers are described in paper [9].

A HP 34420A Hewlett Packard nanovoltmeter was used as a measuring amplifier in the heat power measuring system of the calorimeter. An important advantage of this nanovoltmeter is its link to a computer via RS232 interface which simplifies the problem of building a digital compensation system for heat power measuring. The results of developing such a system were reported elsewhere [1, 10]. They are the ba-

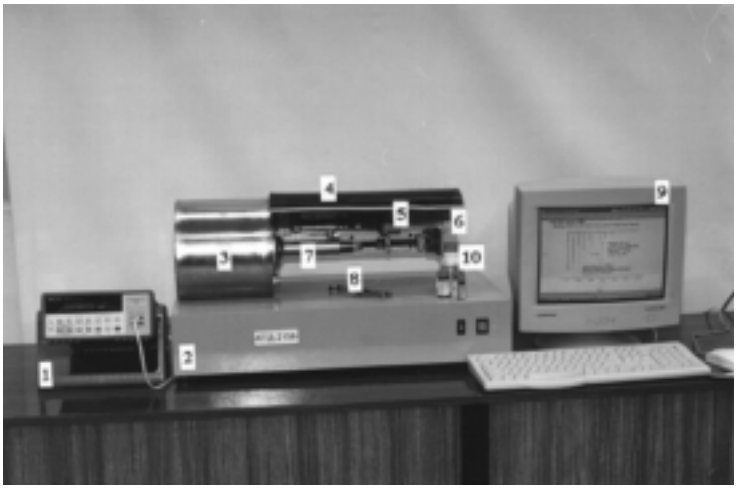

Fig. 5 Differential titration calorimeter KTD-2156: 1 – HP34420A nanovoltmeter; 2 – electronic unit; 3 – calorimetric unit; 4 – protective shield; 5 – stepping motor controlling the syringes; 6 – stepping motor controlling the pistons of syringes; 7 – holders with Hamilton syringes; 8 – feeding syringes; 9 – monitor of control computer; 10 – test preparations

sis of all the digital systems for temperature control providing for thermostatting of the calorimetric chambers. In principle these control systems are close to those described in [11–14].

The calorimetric unit is installed on the base of the instrument in a position permitting a horizontal location of the calorimetric chambers. The mechanisms and the stepping motors of the titration unit are located on a support fixed at a flange of the calorimetric unit which provides for centering of both the chambers and the dispensing needles in syringes located in the holder. The whole titration unit is covered with a protective housing. The base of the instrument permits to arrange reagents and necessary tools on it.

An important advantage of the calorimeter consists in the mode of diffusion mixing due to concentration diffusion implemented in it. At this mode, the calorimeter does not record any heat noise practically arising as a result of needle translation when injecting ligand.

To evaluate the efficiency of the diffusion mixing of the reagents in the chamber using the described method, experiments of mixing the reagents in the chamber with an oscillating dispensing needle were carried out. Tests of the calorimeter with the system Ba^{2+} – 18-Crown-6 gave standard values of ΔH and K in both mixing modes. In the mode of mechanical mixing, the frequency of the needle oscillations was 30 Hz for a span of oscillations of 0.8 mm, the translation speed of the needle along the chamber axis being 2 mm s^{-1}, i.e. at 1 mm distance of translation the needle used to do 15 oscillations. This experiment showed that diffusion mixing provided complete equalization of the reagents concentration. In practice, if both mixing modes give the

same results, the investigator has a possibility to carry out measurements in the mode of diffusion mixing, with maximum sensitivity of the instrument.

The titration calorimeter testing

The most usual tests for trials of titration calorimeters are a process of binding Ba and 18-Crown-6 proposed by Briggner and Wadsø [15] and a process of binding RNase and 2CMP proposed by Wiseman *et al.* [11] and used by Freire *et al.* [6]. Figures 6 and 7 represent the results of testing a KTD-2156 Calorimeter designed and manufactured at the Institute for Biological Instrumentation of the Russian Academy of Sci-

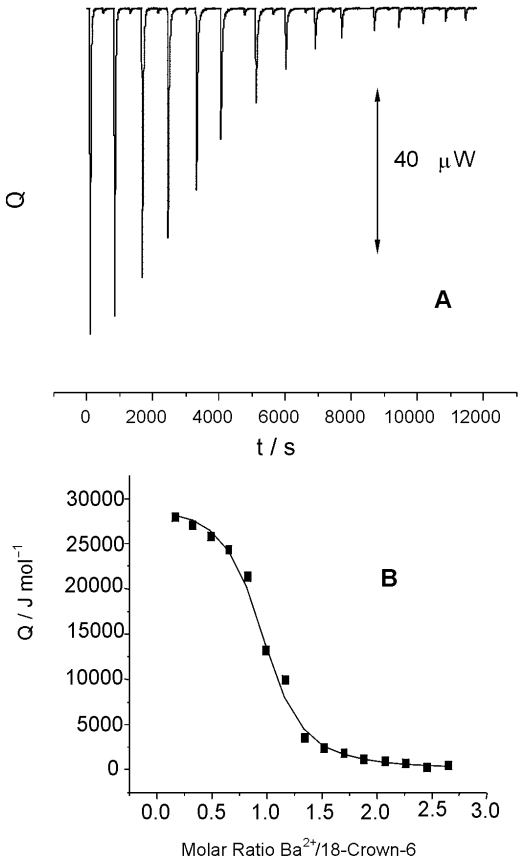

Fig. 6 A – Calorimetric curves of titration of 18-crown-6 with Ba^{2+} at 37°C; B – Plot of the processed data (corresponding to the data of Fig. 5A). The points are experimental and the solid line corresponds to the best-fit curve obtained by least-squares deconvolution. The best values of the fitting parameters are 2724 M^{-1} for K, and –29.62 kJ mol^{-1} for ΔH.
Sample: 78.5 μL of 18-Crown-6 (0.01M), Injection: 1.22 μL of Ba^{2+} (0.1 M)
Mixing: diffusion (without vibration); The reagens were obtained from Sigma

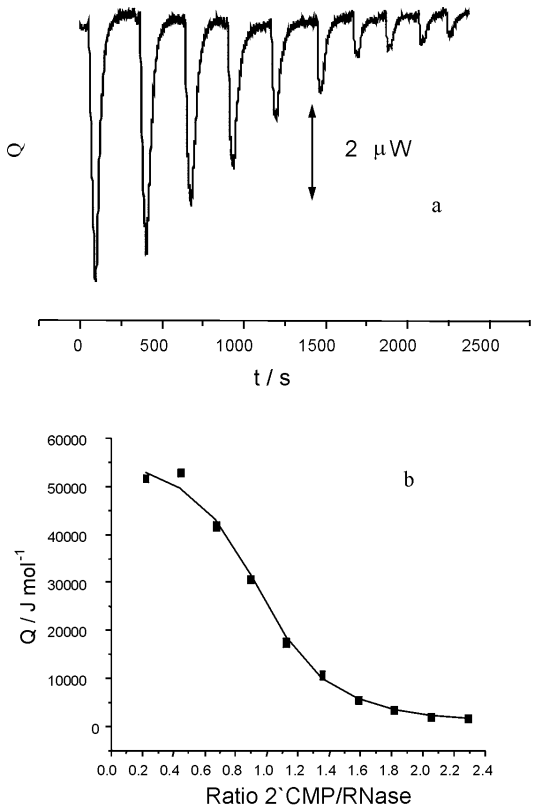

Fig. 7 A – Calorimetric curves of titration of Rnase with 2CMP at 37 °C; B – Plot of the processed data (corresponding to the data of Fig. 8A). The points are experimental and the solid line corresponds to the best-fit curve obtained by least-squares deconvolution. The best values of the fitting parameters are 27425 M^{-1} for K, and –57.562 kJ mol^{-1} for ΔH. Sample: 78.5 μL of RNase (0.651 mM), Injection: 0.4938 μL of 2`CMP (35*0.651 mM); Mixing: needle vibration at 30 Hz, 0.8 mm; Buffer solution: 0.2 M KCl, 0.2 M KAc; pH=5.5; The reagens were obtained from Sigma

ences, Pushchino. To calculate K and ΔH, an algorithm was used which enables to build, on the basis of experimental data, a regressive model according to the least squares method [11].

The data obtained are well compatible with literature data.

Conclusions

Theoretical and experimental investigations proposed in the present paper provided a well-substantiated approach for building a capillary differential titration calorimeter of a new generation ensuring new possibilities:

– Operation with very small amounts of reagents through the use of the volume of the calorimetric chambers of 78.5 μL.

– High precision of the calorimeter due to the separation of the sensitive volume of the calorimetric chambers with an accuracy better than one percent.

– Carrying out measurements at maximum sensitivity because of diffusion mixing of reagents in horizontally located chambers.

Appendix

Determination of the time for ligand concentration equalizing in a sample in the sensitive volume of a cylindrical calorimetric chamber

Theoretical relationship of the time for ligand concentration equalizing along the sample in the sensitive volume of a cylindrical calorimetric chamber was determined under the following conditions.

$V_d = \pi R_0^2 L$, volume of the band (ligand portion), cm^3;

$V_k = \pi R_k^2 L$, sensitive volume of the calorimetric chamber;

R_0, radius of the band, cm;

R_k, internal radius of the calorimetric chamber, cm;

$\rho_1(\tau)$, current average value of the ligand concentration (density) in the volume V_d;

$\rho_2(\tau)$, current average value of the ligand concentration (density) in the volume $(V_k - V_d)$;

$\rho_1 = \rho_n\big|_{\tau=0}$, initial ligand concentration (density) in the band of volume V_d;

$\rho_2 = 0\big|_{\tau=0}$, initial ligand concentration (density) in the volume of the calorimetric chamber outside the band volume $(V_k - V_d)$;

$\rho_k\big|_{\tau=\infty} = \rho_1\big|_{\tau=\infty} = \rho_2\big|_{\tau=\infty}$, final ligand concentration (density) in the sensitive volume of the calorimetric chamber V_k;

The final concentration (density) ρ_k (Fig. 1) was calculated as follows.

The ligand mass is $m_T = V_d\rho_n = V_k\rho_k$. Hence

$$\rho_k = \frac{V_d \rho_n}{V_k} \tag{I}$$

From the mass conservation law: $\rho_2(\tau)(V_k - V_d) = (\rho_n - \rho_1(\tau))V_d$, which gives

$$\rho_2(\tau) = \frac{(\rho_n - \rho_1(\tau))V_d}{(V_k - V_d)} \tag{II}$$

Specific mass flow from the ligand band surface (at $R = R_0$) having the volume V_d due to the concentration diffusion is determined by Fick's law:

$$q = -D_k \left.\frac{d\rho(\tau)}{dR}\right|_{R=R_0}$$

Approximately it can be admitted that:

$$q \approx -D_k \frac{\rho_2(\tau)-\rho_1(\tau)}{R_0} \quad \text{(III)}$$

By substituting $\rho_2(\tau)$ from formula (2) in formula (3) we obtain:

$$q=\frac{D_k}{R_0}\left[\rho_1(\tau)-\frac{(\rho_n-\rho_1(\tau))V_d}{V_k-V_d}\right]=\frac{D_k}{R_0(V_k-V_d)}[\rho_1(\tau)V_k-\rho_n V_d] \quad \text{(IV)}$$

The modification of the ligand mass in volume V_d due to its transport by concentration diffusion can be expressed as:

$$dm(\tau)=m(\tau)-m(\tau+d\tau)=\pi R_0^2 L\rho_1(\tau)-\pi R_0^2 L\rho_1(\tau+d\tau)$$

Since

$$\rho_1(\tau+d\tau)\approx\rho_1(\tau)+\frac{d\rho_1(\tau)}{d\tau}d\tau$$

where τ is the current time, then

$$dm(\tau)=\pi R_0^2 L[\rho_1(\tau)-\rho_1(\tau+d\tau)]=-\pi R_0^2 L\frac{d\rho_1(\tau)}{d\tau}d\tau \quad \text{(V)}$$

On the other hand, the mass variation can be determined as $dm(\tau)=qFd(\tau)$, where $F=2\pi R_0 L$ is the surface area of the band, thus

$$dm(\tau)=\frac{2\pi LD_k}{V_k-V_d}[\rho_1(\tau)V_k-\rho_n V_d]d\tau \quad \text{(VI)}$$

Setting equal Eqs (5) and (6) for $dm(\tau)$, we obtain:

$$-\frac{R_0^2 d\rho_1(\tau)}{d\tau}=\frac{2D_k}{V_k-V_d}[\rho_1(\tau)V_k-\rho_n V_d]$$

hence

$$\frac{d\rho_1(\tau)}{d\tau}=-\frac{2D_k V_k}{(V_k-V_d)R_0^2}\rho_1(\tau)+\frac{2D_k V_d}{(V_k-V_d)R_0^2}\rho_n \quad \text{(VII)}$$

Denoting

$$p=\frac{2D_k V_k}{(V_k-V_d)R_0^2},\ m=\frac{2D_k V_d}{(V_k-V_d)R_0^2}\rho_{in}$$

We have

$$\frac{d\rho_1(\tau)}{d\tau}=-p\left[\rho_1(\tau)-\frac{m}{p}\right]$$

Since $m/p=\rho_k$ is constant, see (I), we obtain

$$\frac{d\left[\rho_1(\tau)-\frac{m}{p}\right]}{d\tau}=-p\left[\rho_1(\tau)-\frac{m}{p}\right]$$

Denoting $\rho_1(\tau) - m/p = y$ we have $dy/d\tau = -py$
Solving this equation: $y = Ce^{-p\tau}$.
If $(\tau)=0$ $\rho_1(\tau)=\rho_n$, then $C=\rho_n-\rho_k$.
Consequently,

$$\rho_1(\tau) - \rho_k = (\rho_n - \rho_k)e^{-p\tau} \quad \text{(VIII)}$$

Taking the logarithm of Eq. (VIII) we find the expression for τ:

$$\tau=\frac{1}{p}\ln\frac{\rho_n-\rho_k}{\Delta\rho} \quad \text{(IX)}$$

where $\Delta\rho = \rho_1(\tau)-\rho_k$.

Using formula (I) we obtain $\rho_{in}- \rho_f=\rho_f[V_k/V_d-1]$. Substituting this expression and the above obtained value of p to formula (IX) we obtain the formula for the time of ligand concentration (density) equalizing in the sample that is in the sensitive volume of the calorimetric chamber

$$\tau=\frac{R_0^2}{2D_k}\left(1-\frac{V_d}{V_k}\right)\ln\left[\frac{1}{k}\left(\frac{V_k}{V_d}-1\right)\right] \quad \text{(X)}$$

where $k=\Delta\rho/\rho_k$.

Equation (X) comprises the value of the ligand diffusion factor in the sample. We could not find a reference value for the diffusion factor of the ligand 0.5 mM NADH in a sample of 4.28 μM LDH. That is why the diffusion factor was determined using the time for diffusion concentration equalizing of a ligand in a sample contained in an optical cell of a spectrophotometer. Using the theoretical ratio $k=\Delta\rho/\rho_k$ the authors determined the diffusion factor on the base of time characteristics of spectrophotometric readings.

Determination of the diffusion factor by the time of ligand concentration equalizing in a sample in a spectrophotometric optical cell

The calculation was performed on the model with the following conditions (Fig. 8).

$V_T = \pi r_{sam}^2 L$ is the volume of ligand band injected into an optical cell.

Since $2r_{sam}\approx d$ ($2r_{sam}$=0.098 cm, d=0.1 cm), to simpify of the task we admit that the ligand is distributed inside a parallelepiped with the thickness $d_{sam}=V_T/dL$.

$V_{opt}=V_T+V_2=dHL$, internal volume of the optical cell;

$V_2=d(H-d_{sam})L$, internal volume of the optical cell without the volume of the ligand band;

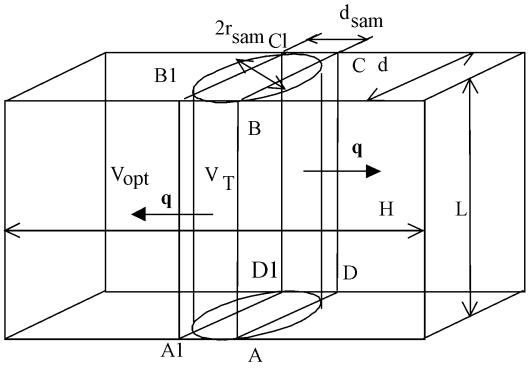

Fig. 8 Titrant portion as a band in a spectrophotometric optical cell of hight *L*

$\rho'_1=\rho'_{in}\big|_{\tau=0}$, initial ligand concentration (density) in the band of volume V_T;

$\rho_2=0\big|_{\tau=0}$, initial ligand concentration (density) in the internal volume of the optical cell V_2 outside the band;

$\rho'_f\big|_{\tau=\infty}=\rho'_1\big|_{\tau=\infty}=\rho'_2\big|_{\tau=\infty}$, final ligand concentration (density) in the internal volume of the optical cell V_{opt};

Using a procedure analogous to the above for the calorimetric vessel, a balance equation similar to Eq.(VII) is obtained with the parameters expressed as follows

$$p'=\frac{4D_kV_{opt}}{(V_{opt}-V_T)d_{sam}^2},\ m'=\frac{4D_kV_T}{(V_{opt}-V_T)d_{sam}^2}\rho'_{in},\ \frac{m'}{p'}=\rho'_f$$

The solution of this equation is similar to that of Eq. (VII), the value $\rho'_1(\tau)$ for the optical cell being represented by expression (XI):

$$\rho'_1(\tau)-\rho'_f=(\rho'_{in}-\rho'_f)e^{-p\tau} \qquad \text{(XI)}$$

From formula (XI), in the way analogous to deducing formula (X), we obtain an expression for D_k

$$D_k=\frac{d_{sam}^2}{4\tau_{exp}}\left(1-\frac{V_T}{V_{opt}}\right)\ln\left[\frac{1}{k'}\left(\frac{V_{opt}}{V_T}-1\right)\right] \qquad \text{(XII)}$$

where τ_{exp} is the experimentally found time (in seconds) of ligand concentration (density) equalization in the sample throughout the internal volume of the optical cell; $k'=\Delta\rho'/\rho'_f$.

The value $D_k=1.75\cdot10^{-5}$ cm^2 s^{-1} is determined from Eq. (XII) for

$k'=0.05$;

$V_T=33\cdot10^{-3}$ cm^3;

$V_{opt}=dHL=0.1\cdot1\cdot4.4$ cm^3=0.44 cm^3;

$d_{sam}=V_T/dL=33\cdot10^{-3}/0.1\cdot4.4=0.075$ cm.

Substituting the obtained D_k value in Eq. (X), we have calculated the time of diffusion equalization of the ligand concentration in the sample contained in a cylindrical calorimetric chamber τ=37 s for the inside diameter of the capillary of the calorimetric chamber of 0.12 cm (R_k=0.06 cm and V_k=78.48·10^{-3} cm^3); the capillary length L=5 cm; the ligand band diameter is 0.03 cm ($R_0=\sqrt{V_d/\pi L}$=0.149·10^{-1} cm and V_d=3.5·10^{-3} cm^3 for the ligand band length L=5 cm); D_k=1.75·10^{-5} cm^2 s^{-1}; k=0.05.

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