Thermochimica Acta, 33 (1979) 7—17

APPLICATIONS OF DIFFERENTIAL SCANNING CALORIMETRY TO ENZYME KINETICS

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(Received 11 September 1978)

ABSTRACT

Differential scanning calorimetry has been investigated for its application to the experimental study of the temperature dependence of the kinetics of enzyme—substrate reactions. A reaction cell was developed which allows mixing of enzyme and substrate solutions directly in the calorimeter. The device was used to study the zero-order reaction of acetylcholinesterase with acetylcholine and the first-order reaction of α-chymotrypsin with *N*-acetyl-L-alanine methyl ester. The reaction cell is found to be satisfactory in the isothermal mode for both first- and zero-order reactions and in the scanning mode for the zero-order reactions but not for the first-order reaction. Limitations of the design are described for general enzyme kinetic studies.

INTRODUCTION

The elucidation of the mechanism of catalysis for enzyme reactions has been the subject of biochemical interest for many years. Researchers have found that the systematic variation of the reaction temperature can yield useful information concerning both the activation enthalpy and entropy of the reaction [1]. These activation parameters provide data which are relevant to the study of the mechanism of the enzyme's action [2—4]. Similar use of activation parameters can be found in the kinetic studies of the thermal stability of enzymes [5]. Classically, these activation parameters are obtained by carrying out a series of isothermal reactions over a moderate temperature range (20—30°C) where the rate of reaction is usually followed spectrophotometrically. The data are then analyzed for the activation entropy and enthalpy by means of an Arrhenius plot [1].

Rather than carrying out a series of isothermal reactions, it would presumably be much more efficient to carry out a single DSC experiment where the temperature is linearly varied over the desired range. In principle, a DSC experiment could yield the same results as the isothermal studies, providing that the reaction produces or consumes a measurable amount of heat. It

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should be noted that, in general, calorimetric methods complement spectrophotometric techniques in that reaction-generated heat can be detected in many cases where the substrate or product does not absorb in the visible or UV regions. One further advantage of DSC is that the enthalpy of reaction can be obtained directly from the area under the DSC peak.

Differential scanning calorimetry as developed during the last few years has a distinct advantage in that the sample sizes are typically small (10—100 μl for the Perkin-Elmer instruments). Thus, only minute amounts of often expensive enzymes are needed for experimental purposes. Naturally, when such small sample volumes are used, high calorimetric sensitivity is required to detect the small amount of reaction-generated heat.

In light of the above advantages of investigating enzyme reactions by DSC, an examination of the possible general application of DSC to enzyme—substrate reactions was undertaken. To date there has only been one attempt at studying an enzyme—substrate reaction by DSC [6]. Cassel [6] briefly investigated the catalase—peroxide reaction by DSC where enzyme and substrate were pre-mixed on a cold plate, rapidly frozen, sealed in a 15-μl "volatile" sample pan, and then temperature-scanned from below the freezing point of the mixture. The reaction began immediately upon melting of the aqueous sample and thus the associated heat was superimposed on the solvent melting peak. Analysis of this data was not carried out for the temperature dependence of the rate parameters. We have previously reported a theoretical simulation of the heat and mass transfer processes which take place in solution phase DSC [8]. This work was used to establish the guidelines for the experimental conditions employed in the present study.

EXPERIMENTAL

Apparatus

A Perkin-Elmer differential scanning calorimeter (model DSC-II), equipped with a full range calorimeter sample holder assembly, was used throughout this study. The DSC output was recorded on a Leeds and Northrup Speedomax recorder. The DSC-II was set up for subambient operation with a low temperature heat sink bath and cold finger. Depending on the lowest sample temperature needed, one of the following low temperature baths was employed: dry-ice/acetone (240 K) or dry-ice/ethylene glycol/water (265 K). The temperature of the bath must be 30—50 K below the desired lowest temperature of the sample to insure proper operation of the instrument. The nitrogen purge gas pressure was maintained at 20 psi.

Sample injections for the flow-mixing design (see Fig. 1) utilized a Syringe Microburet (model SB2, Micrometric Instrument Cc., Cleveland, Ohio) which was equipped with a 10-μl fixed-needle Hamilton syringe. Reagent No. 1 was loaded with a Pressure-Lok 25-μl syringe. Epotek 360 epoxy (low viscosity), Epoxy Technology, Inc., Watertown, MA, was used to make the seal between the Teflon tube and the reaction chamber tube. Lite-wall Teflon tubing was obtained from Zeus Industrial Products, Inc., Raritan, N.J.

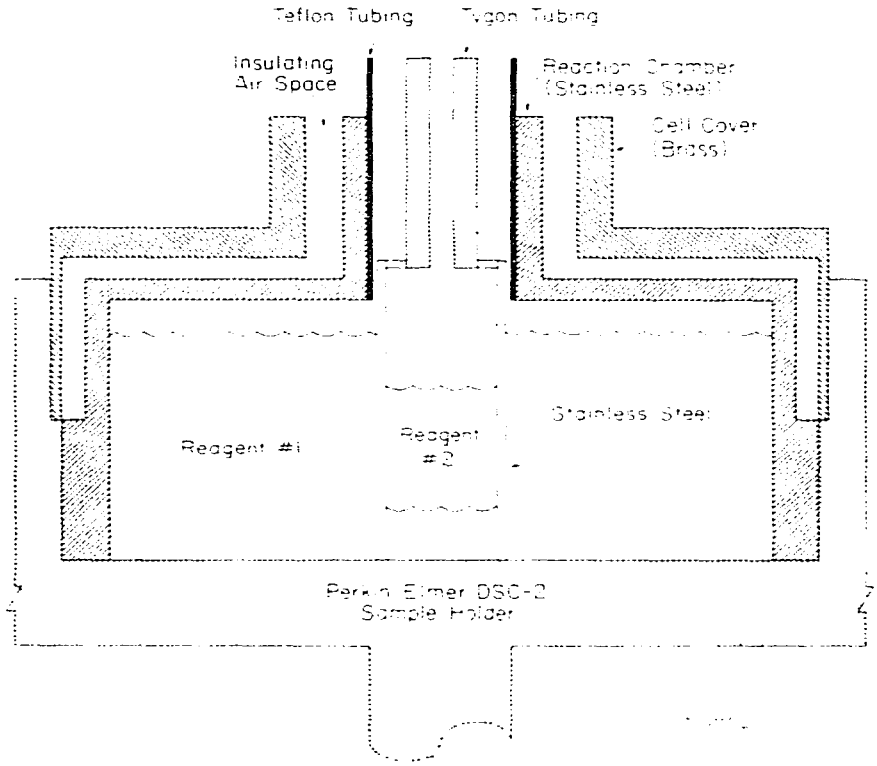


Fig. 1. Flow-mixing cell design.

All enzyme and substrate solutions were prepared daily. The α-chymotrypsin (α-CT), acetylcholinesterase (AC), and acetylcholine bromide (ACB) were obtained from Sigma Chemical Co. and were stored desiccated below 0°C, while the *N*-acetyl-L-alanine methyl ester (AAME) was obtained from Chemalog and stored at 0—5°C. ACB solutions were prepared with 1 M tris-hydroxymethylamino methane (THAM), 1 mM EDTA, pH = 8.0, to concentrations ranging from 10 to 15 mg ml^{-1} protein, 235 units mg^{-1} solid protein. ACB solutions were also prepared with 1 M THAM, 1 mM EDTA, pH = 8.0, but at concentrations ranging from 0.097 to 0.16 M ACB. Calcium was found to stabilize the high protein concentrations in the α-CT study, thus, α-CT was prepared at 30—40 mg ml^{-1}, 44 units mg^{-1} solid protein, and AAME was prepared at 1.6—1.8 M nominal concentrations in 1 M THAM, 100 mM $CaCl_2 \cdot 2\,H_2O$, 1 mM EDTA at pH = 8.0. Slight turbidity was noted in all AAME solutions.

RESULTS AND DISCUSSION

It is important to realize that there is a fundamental problem associated with the study of enzyme reactions by DSC. In contrast to the usual single component samples in DSC, such as a solid polymer sample or a protein solution, one must now deal with a two-reagent system rather than a one-reagent

system. One cannot simply encapsulate an enzyme—substrate mixture and place it in the DSC for a temperature scan. As soon as the two reagents mix, chemical reaction takes place and, depending on the amounts of enzyme and substrate, and the temperature at which they are maintained during encapsulation, the entire reaction may be complete even before a temperature scan can be initiated. In any case, a significant portion of chemical reaction will have taken place before the experiment is begun, unless the start of the reaction can be controlled. One possible means by which this may be accomplished is to mix the two solutions directly in the DSC sample holder at a pre-determined temperature and to immediately observe the enzyme reaction. The flow-mixing approach employed here uses air-pressure to force the mixing of the enzyme and substrate in a specially designed reaction cell. A previous study on the heats of mixing of several organic solvents used a syringe to inject samples into a solvent in the DSC sample holder [7]. This approach is not suitable for the present studies due to the typically very small amounts of heat generated during an enzymatic reaction.

Flow-mixing cell design

The flow-mixing cell design utilized in this study is shown in Fig. 1. Preliminary designs have been described elsewhere [10]. Attached to the stainless steel inner chamber is a Lite-wall (TM) Teflon tube which extends several centimeters up and out of the low-temperature heat sink environment. Thus, 25—75 μl aqueous samples of reagent No. 1 may be loaded directly with a syringe. External to the reaction cell, reagent No. 2 is loaded by either direct placement of 1—3 μl of the desired sample in the injection tube or the injection tube is placed down into the desired solution and 1—3 μl is "sucked" up into the tube with a 10 μl fixed-needle syringe attached to the other end of the Tygon tube. A small air bubble (0.5 μl) is introduced into the tip of the tube to avoid premature mixing of enzyme and substrate. This injection tube assembly loaded with reagent No. 2 is slipped down the Teflon guide tube until it enters the reaction chamber and rests near the base of the sample holder as illustrated in Fig. 1. After thermal equilibrium has been achieved, the 10 μl syringe is actuated and reagent No. 2 is forced into No. 1, thereby initiating the chemical reaction. The cell is cleaned and dried by aspiration after a line which is connected to a vacuum is inserted into the Teflon guide tube. A dilute soap solution, water, and acetone, in that order, are delivered to the cell via a syringe. The cell is dried by sweeping it with dry nitrogen. This design uses only a silicone grease seal between the sample holder and the reaction chamber. Extensive cleaning of the cell can remove this seal, resulting in an unstable baseline or, worse, leakage of wash- or rinse solutions onto the electronics of the sample holder. Frequent monitoring of this seal is therefore important.

Several basic advantages accrue from this cell design. (1) Both reagents are in close contact with each other and with the sample holder base, which means that temperature mis-match between samples is small, thus low injection blank signals are possible. (2) The design is very simple and uses only one permanently attached Teflon tube which is centered in the holder. Since

the manufacture of such small and intricate cells is usually difficult, the simplicity of the design means that fewer difficulties should be encountered in the machining process. This in turn implies that reproducibility between manufactured cells should be much better and matching of sample and reference cells is less of a problem. Matching of sample and reference, both in heat capacity and physical dimensions, is very critical in achieving a stable, flat, and reproducible baseline in the scanning mode. (3) This design utilizes an outer "insulating" cover which considerably reduces vaporization and condensation noise associated with volatile samples such as aqueous solutions. (4) Due to the simplicity of design, sample handling and cell cleaning can be accomplished without removing the cell from the sample holder. This improves baseline reproducibility. (5) The amount of material used in the design has been minimized so that thermal response times are relatively fast ($\tau \sim 7$ to 10 sec first-order time constant).

The details of cell performance, i.e., baseline noise, baseline drift, response time, injection blank, and calibration studies, are available elsewhere [10].

The major disadvantages of the cell design are: (1) one is limited to ~320 K as the upper limit of a temperature scan for an aqueous sample due to volatilization of water which causes baseline drift and noise; (2) since no means are provided for the physical mixing of enzyme and substrate in the cell, one must rely upon natural convection and vibrations to effect mixing. The first-order time constant (τ) has been experimentally measured at ~20 sec which means that after ~100 sec (5 τ) the two solutions will be mixed with better than 99% completeness. One is therefore limited to relatively slow reactions lasting longer than 2—3 min in order to obtain undistorted data. (3) Baseline noise due to vaporization and condensation effects is a function of temperature and can be quite large $(\sim 10\ \mu\text{cal sec})^{-1}$ at higher temperatures.

Applications to enzyme kinetics

In order to determine the utility of the flow-mixing cell design for the study of enzyme kinetics, we examined both a zero-order and a first-order enzyme reaction which were well studied. Unfortunately, very few common enzyme reactions which strictly follow Michaelis—Menten kinetics can be carried out at high substrate concentration. In addition, very few substrates have sufficiently high water solubility to produce sufficient heat to make the baseline noise relatively unimportant.

The enzyme—substrate reaction of acetylcholinesterase (AC) with acetylcholine (ACB) was selected for study in the zero-order (substrate saturation) kinetic region due to its low Michaelis constant $K_M \sim 10^{-4}$ M, high substrate solubility, and high enzyme activity [9]. The kinetics of the reaction are not strictly Michaelis—Menten in two respects. First, the accepted mechanism is formulated as two basic steps in contrast to simple Michaelis—Menten kinetics

$$EH + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EHS \xrightarrow{k_3} ES' + ROH$$

$$ES' + H_2O \xrightarrow{k_4} EH + CH_3COOH$$

The first step involves the formation of the acetyl-enzyme (ES′) species, while the second involves the hydrolysis of this species. For acetylcholine (ACB) it has been shown that the first step is rate limiting at lower temperatures while the second is rate limiting at higher temperatures. Since neither step predominates totally over the temperature range 5—35°C, a non-linear Arrhenius plot is obtained with an approximate activation energy of 0—2.5 kcal $mole^{-1}$ for the second step, i.e., hydrolysis of the acetyl-enzyme species, at higher temperatures, and an approximate lower temperature activation energy for the first step, i.e., formation of the acetyl-enzyme species, of 14—19 kcal $mole^{-1}$ [4]. The second kinetic complication is substrate inhibition of AC by high concentrations of ACB [11].

Both the above kinetic complications have been investigated by DSC with satisfactory results. A series of isothermal mode DSC initial rate experiments were carried out to demonstrate that one can reproduce the literature studies. A typical DSC trace for this isothermal zero-order reaction is shown in Fig. 2. For a pure zero-order reaction, one should expect a constant rate of heat production as demonstrated in the trace when one allows sufficient time for mixing and corrects for baseline drift. The baseline drift in these studies is most likely due to loss of water vapor up the Teflon tube. The exothermic displacement of the reaction signal from the baseline is directly proportional to the rate constant at the given temperature. The experimental Arrhenius plot of Fig. 3 obtained from a series of isothermal runs is in excellent agreement with the literature values for the limiting activation energies states above. Figure 4, which illustrates highly reproducible Arrhenius plots from experiments at three different scan rates represents an attempt to obtain the same results in the DSC scanning mode. Ideally, all three scan rates should have yielded the same results as the isothermal experiments. This lack of agreement between scanning and isothermal results can be shown to be

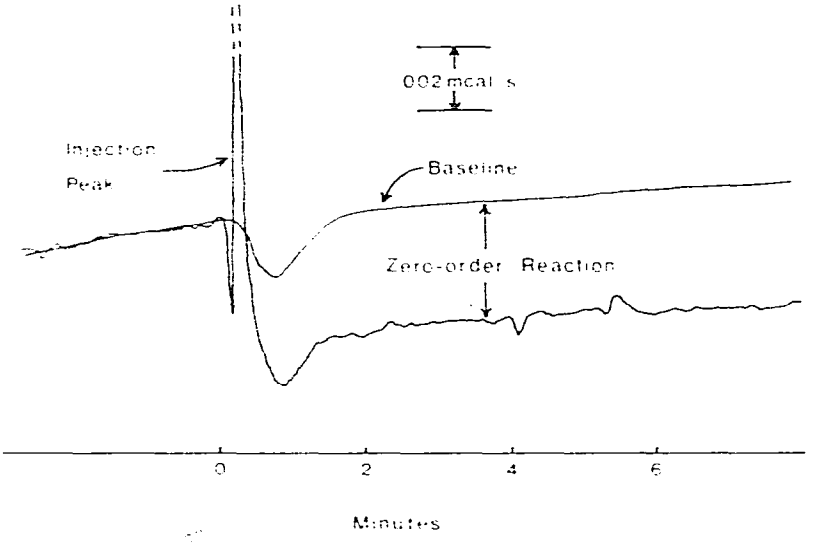
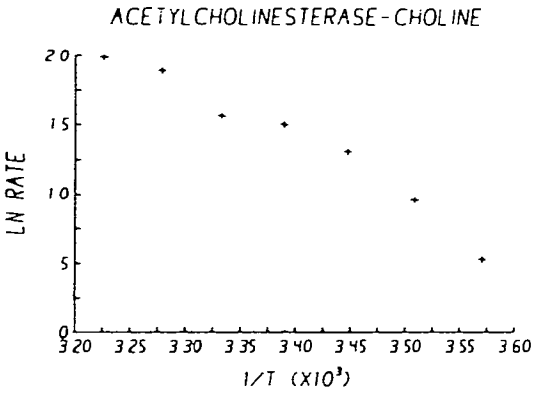


Fig. 2. Rate of heat output vs. time for the isothermal zero-order reaction of acetylcholinesterase with acetylcholine: 2 μl of 11.8 mg ml^{-1} of acetylcholinesterase was injected into 50 μl of 0.097 M acetylcholine bromide at 295 K.



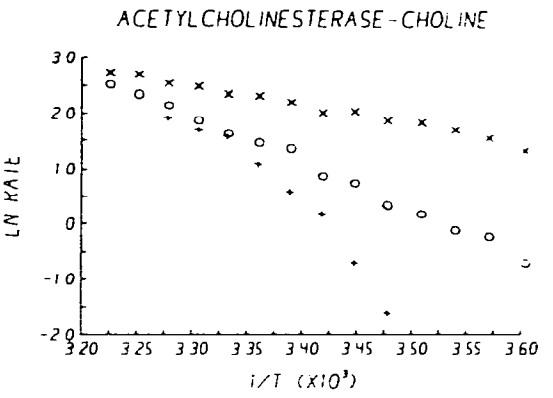


Fig. 3. Arrhenius plot for acetylcholinesterase obtained from a series of isothermal reactions. All conditions are the same as given in Fig. 2 except the initial temperature was varied.

Fig. 4. Arrhenius plots for acetylcholinesterase obtained from DSC scans. Conditions are the same as in Fig. 2 except the cell temperature was scanned at the following rates: 5 K min^{-1} (×); 2.5 K min^{-1} (○); 1.25 K min^{-1} (+).

due to substrate inhibition rather than improper DSC experimental technique. An apparent inhibition constant, K_I, of ~10 mM has been demonstrated previously [12] and is nearly an order of magnitude below our normal working concentrations of ~100 mM acetylcholine. One should therefore expect significant substrate inhibition at this concentration. Wilson [11] has postulated that substrate inhibition should increase with increasing temperature. Our evidence lends support to this theory and goes on to quantitate the temperature dependence. Figure 5 illustrates an isothermal DSC experiment at 310 K where substrate inhibition is indicated by the increase in slope of the usual flat zero-order reaction trace. As the substrate inhibits the enzyme reaction, it causes a decrease in the rate of heat production. We have been able to experimentally show that the calorimetric slope of this inhibition process is a strong function of temperature and can be related to the temperature dependence of the rate of inhibition. An Arrhenius plot of this inhibition process is illustrated in Fig. 6. It is not the purpose of this investigation to enter into speculation on the kinetic significance of this non-linear Arrhenius plot, but only to demonstrate the utility of DSC for obtaining data on the temperature dependence of enzyme kinetic processes. Our point is that static and dynamic DSC measurements yield the same results.

The enzyme reaction of α-Chymotrypsin (α-CT) with *N*-acetyl-L-alanine methyl ester (AAME) was selected for the study of a first-order kinetic process since it has a high Michaelis constant, K_M ~ 600 mM, high substrate solubility, and high enzyme activity [13]. A representative example of an isothermal DSC trace of the α-CT-AAME reaction at 315 K is illustrated in Fig. 7. After mixing is complete a first-order reaction process is observed from which the rate constant may be obtained by evaluating the slope of a plot of the logarithm of the relative rate of reaction against time. A series of

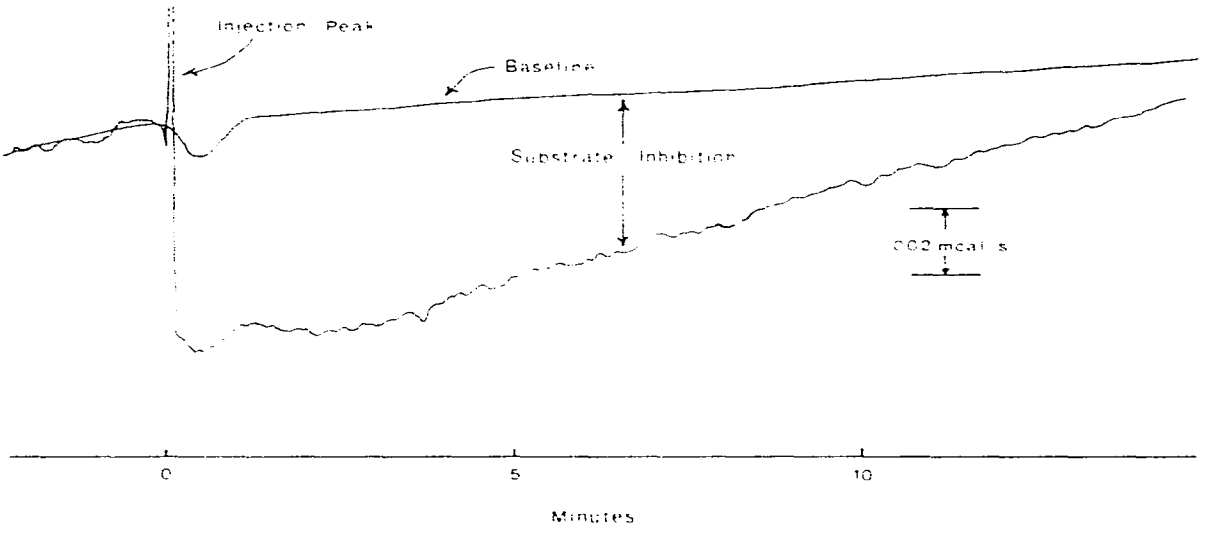


Fig. 5. Heat output vs. time for the isothermal zero-order (substrate inhibited) reaction of acetylcholinesterase with acetylcholine: All conditions are the same as in Fig. 2 except the initial temperature is 320 K.

isothermal experiments yielded a linear and reproducible Arrhenius plot (shown in Fig. 8) with an activation energy of 10.3 kcal mole^{-1} and a standard deviation of 0.9 kcal mole^{-1}. Although no direct comparison of activation energy was found in the literature for this substrate, it was within ±1 kcal mole^{-1} of the activation energies of four other related substrates suggesting that the value of 10.3 kcal mole^{-1} is very reasonable [2]. An attempt to obtain this same data directly from a DSC scan of the reaction was thwarted by uncertainties in establishing the baseline. Figure 9 illustrates a typical temperature scan of the α-CT-AAME reaction mixture. Although the fluctuations in the baseline scan are reproducible to ~3% full scale, (200 μcal sec^{-1}) they make analysis of the data difficult. We believe that these fluctuations are related to the control system of the DSC and the presence of

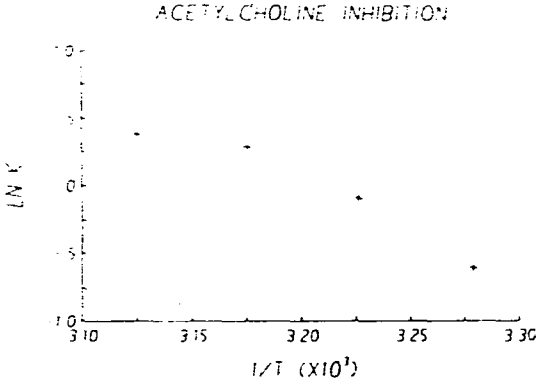


Fig. 6. Arrhenius plot of substrate inhibition process for the acetylcholinesterase—acetylcholine reaction.

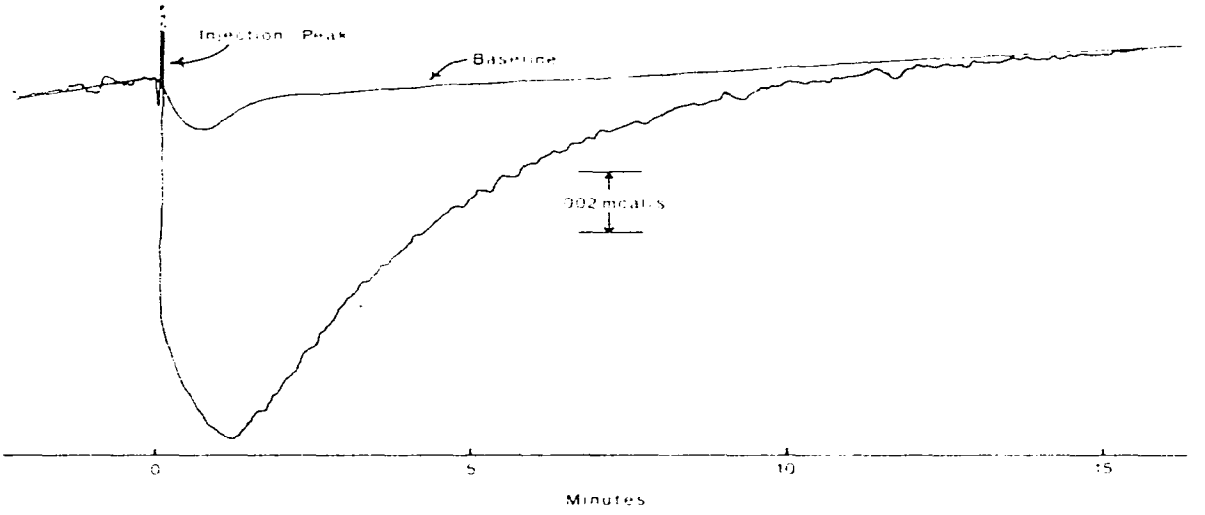


Fig. 7. Heat output vs. time for the isothermal first-order reaction of α-chymotrypsin with *N*-acetyl-L-alanine methyl ester: 2 μl of 1.8 M AAME was injected into 50 μl of 47 mg ml^{-1} α-CT at 315 K.

absorbed water in our reference cell. An additional problem is the significant baseline shift (>20 μcal sec^{-1}) on re-scanning the baseline which is probably due to loss of water vapor out the Teflon tubes. This phenomenon increases with increasing temperature so that scans above 310 K are not advisable in terms of baseline reproducibility. The reader should be reminded that in analyzing a first-order reaction by DSC in the scanning mode, one needs an accurate value for the total area under the trace, a calculation which is highly dependent on baseline reliability. This is in contrast to the zero-order case where the area under the trace is not required in the analysis. In order to complete the investigations on the α-CT-AAME reaction, the theoretical findings of previous work were applied to this system [8].

In addition to the experimental problem of baseline reproducibility, random experimental errors related to slow scan rates should be quite severe in

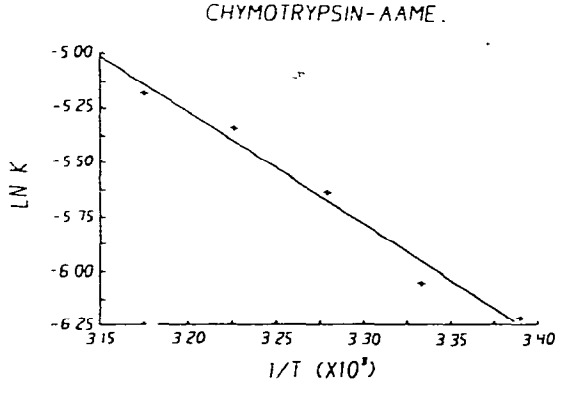


Fig. 8. Arrhenius plot of first-order reaction of α-chymotrypsin with *N*-acetyl-L-alanine methyl ester. Conditions same as in Fig. 7 except initial temperature was varied.

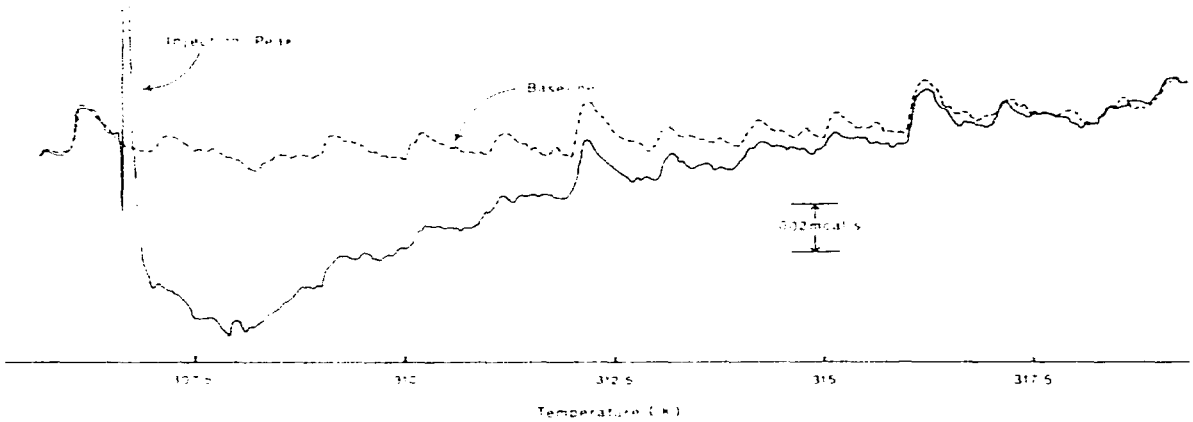


Fig. 9. DSC scan of the first-order reaction of α-chymotrypsin with *N*-acetyl-L-alanine methyl ester. Conditions same as in Fig. 7 except scan rate was 0.625 K min^{-1}.

the investigation of the α-CT-AAME reaction. An attempt was made to increase the scan rate in order to alleviate this problem, however, it necessitated lowering the initial temperature so that the reaction would be complete before reaching 320 K, the upper practical scanning limit. This change had the effect of reducing the average reaction heat to such a degree that the scan was indistinguishable from a simple baseline shift. Clearly DSC with our cell design is severely limited in its application to the above first-order reaction in the scanning mode.

CONCLUSION

An evaluation of DSC as applied to enzyme kinetic studies is appropriate at this point. With the device presented here reliable scanning data for a first-order kinetic reaction can be obtained with a total reaction generated heat of approximately 100 mcal. For a 50 μl sample of substrate and a heat of reaction of 10 kcal $mole^{-1}$, the requisite substrate concentration is 0.5 M. This is an extremely high substrate concentration and cannot usually be obtained due to solubility problems. There are somewhat less severe restrictions for a zero-order reaction since baseline reproducibility is not as critical as in the first-order region. However, we estimate that a 0.1 M substrate concentration will be needed for a zero-order reaction. This is difficult to achieve. Since most enzyme kinetic studies are carried out in the 1—10 mM range, unless a factor of ten to one hundred improvement in baseline noise and reproducibility can be achieved, only limited applications of DSC to enzyme kinetics will be possible. The above estimates on minimum substrate concentration assumed sufficient enzyme activity which may not always be the case. For both the zero and first-order case an average baseline deflection due to chemical reaction at the highest temperature of the scanning interval should be approximately 100 μcal sec^{-1}. For a heat of reaction of 10 kcal $mole^{-1}$ this requires an enzyme activity of 0.6 units (μmoles substrate

min^{-1}). Although this is not very large, when one realizes that this activity must be contained in 2—50 μl aqueous solution, the actual enzyme concentrations required may be as high as 30—40 mg ml^{-1} protein. Many enzymes are not soluble to this extent. Improvement on baseline stability would also ease this problem.

The above investigations have shown that DSC may be applied to only a limited number of enzyme reactions with satisfactory results. However, the isothermal capabilities of DSC should not be underestimated since it has been demonstrated in this study that highly reliable results may be obtained in this manner.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Science Foundation (CHE 75-19412).

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