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# THE ENTHALPIMETRIC DETERMINATION OF INHIBITION CONSTANTS FOR THE COMPETITIVE INHIBITION OF SERUM CHOLINESTERASE BY MORPHINE, QUININE AND PROCAINE

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A method is described for the enthalpimetric determination of inhibition constants based on a previously reported procedure for the determination of $K_m$, using the integrated Michaelis–Menten equation. The inhibition of the cholinesterase-catalyzed hydrolysis of butyryl-choline by morphine, quinine and procaine is chosen as a model. The results compare favourably with Lineweaver-Burk data.

The determination of enzymatic inhibition constants has significance in several areas of chemistry. Many antibiotics and some other drugs produce their biological effects by acting as enzyme inhibitors. Their selection, although still made on empirical grounds, is based on their inhibitory action of a metabolic step more critical to an infective cell or virus than to the host. A rational approach to chemotherapy would therefore be the identification of a strategic enzymatic reaction followed by a synthesis of suitable inhibitors. In this context, the magnitude of $K_i$, the dissociation constant of the enzyme-inhibitor complex, becomes important. In purely analytical applications, knowledge of the magnitude of $K_i$ allows predictions regarding the analytical sensitivity of an inhibition-based analysis since it reflects the "potency" of the inhibitor with respect to a particular enzyme-catalyzed reaction.

Enthalpimetric methods have been reported for the determination of biochemical kinetic parametres. Beezer *et al.* [1] discussed the advantages and limitations of applying flow calorimetry to the determination of $K_m$, the Michaelis constant, for the urea-urease system. Tangential extrapolation of kinetic direct injection enthalpograms has also been used for the determination of $K_m$ [2]. Lineweaver-Burk "double-reciprocal" plots have typically been employed to obtain the value of $K_m$ from such data. The limitations of such plots have been discussed at length elsewhere [3, 4].

An alternative and more convenient approach to the determination of $K_m$ is to generate a single enthalpimetric progress curve and then fit the data to the integrated Michaelis–Menten equation. This principle has been used successfully for the enthalpimetric determination of $K_m$ for the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester [5]. In the present report it is shown that a similar procedure can be used for the enthalpimetric determination of $K_i$, the

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inhibition constant. This method requires one experiment if $K_m$ is already known, two experiments if not.

The competitive inhibition of serum cholinesterase by morphine, quinine and procaine have been chosen as model systems. It was shown in a separate study [6] that the competitive inhibition mechanism is involved in these reactions.

## Theoretical principles

The Michaelis–Menten equation for an uninhibited enzyme-catalyzed reaction can be written,

$$-\mathrm{d}S_t/\mathrm{d}T = V_{max}S_t/(K_m + S_t) \tag{1}$$

where $S_t$ is the number of moles of substrate at time $t$. Integrating between the limits $S = S_0$ and $S_t$ and $t = 0$ and $t$ gives

$$(S_0 - S_t)/t = V_{max} - [K_m \ln S_0/S_t(1/t)] \tag{2}$$

where $S_0$ is the initial number of moles of substrate. Thus a plot of $(S_0 - S_t)/t$ *versus* $(1/t)\ln S_0/S_t$ will be linear with an ordinal intercept of $V_{max}$ and a slope of $-K_m$. It can easily be shown that for a competitively inhibited enzymatic reaction, Eq. 2 becomes,

$$(S_0 - S_t)/t = V_{max} - [K_m(1 + {}^{[I]}/K_i) \ln S_0/S_t(1/t)] \tag{3}$$

The slope is now given by $-K_m(1 + {}^{[I]}/K_i)$ where [I] is the concentration of the inhibitor. Evidently, $K_m$ and $K_i$ can be determined from successive progress curves monitoring reactions in the absence and presence of inhibitor respectively.

The heat output at any instant during the course of a reaction can be represented by

$$q_t = (S_0 - S_t)\Delta H \tag{4}$$

where $\Delta H$ is the enthalpy of reaction (J mol$^{-1}$). Alternatively, a purely calibrative approach can be used if the relationship,

$$(S_0 - S_t) = (q_t/q)S_0 \tag{5}$$

is utilized. In Eq. 5, $q$ represents the total heat effect of the reaction going to completion (see Fig. 1). In either case it is apparent that the dependent variable in Eq. 3 (or 2), $S_t$, can be determined directly from an enthalpimetric progress curve. Equation 5 was utilized in the present study. It transpires that the enthalpy associated with the enzymatic hydrolysis of butyrylcholine per se is not sufficient to monitor the reaction. In the presence of Tris buffer, however, the concurrent protonation of Tris amplifies the heat change to acceptable proportions [7].

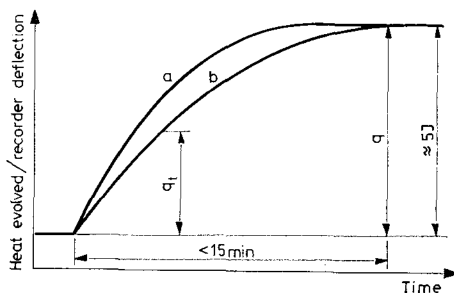


Fig. 1. Typical enthalpimetric progress curves a) $K_m$ data, in the absence of inhibitor and b) $K_i$ data in the presence of inhibitor. Both curves are corrected for heat losses

## Experimental

### *Reagents and apparatus*

Type IV horse serum cholinesterase (E.C.3.1.1.8; nominal activity 15 IU mg$^{-1}$ protein) and butyrylcholine iodide (99%) were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). The alkaloids (pharmaceutical grade) were gifts from R. H. McKeown, Department of Pharmacy, University of Otago, Dunedin, New Zealand. Details of the enthalpimetric instrumentation can be found elsewhere [5].

### *Procedure*

In order to determine $K_m$, approximately 0.5 cm$^3$ of solution containing 80 IU enzyme was injected into 100 $\mu$mol of substrate dissolved in 10.0 cm$^3$ Tris buffer (0.2 mol cm$^3$ pH 8.0). Both solutions were pre-equilibrated to the thermostat bath temperature of 25.0° (short term stability $\pm$0.0002°) as evidenced by an isothermal baseline. The enthalpimetric progress curve was monitored on a strip-chart potentiometer and the data treated according to Eq. 2. A similar experimental procedure was adopted for the determination of $K_i$ with the exception that the substrate solution was allowed to equilibrate with a known amount of inhibitor prior to injection. Characteristically for reversible inhibition, equilibration time had no observable effect on the data. The amount of inhibitor was adjusted so that the final concentration (in the cell) was close to the expected value of $K_i$. The data were plotted according to Eq. 3. A selection of data treated according to the integrated Michaelis – Menten equation was compared with results obtained using the conventional Lineweaver-Burk approach. The enthalpimetric measurement of initial reaction rates required for a double reciprocal plot necessitated a change in reagent parameters. In these experiments, 1.0 cm$^3$ of solution containing 5.0 IU enzyme was equilibrated with 9.0 cm$^3$ of Tris buffer and 1.0 cm$^3$ of inhibitor solution. Reactions were initiated by injection of 0.5 cm$^3$ of substrate solution

containing between 20 $\mu$mol and 100 $\mu$mol of butyrylcholine. Gravimetrically calibrated gas-tight syringes with Chaney adaptors gave the required precision for injection of known amounts (mol) of substrate.

## Results and discussion

The graphical determination of $K_i$ according to the procedure described requires knowledge of the magnitude of $K_m$. Results for the enthalpimetric determination of $K_m$ for the cholinesterase-butyrylcholine system are presented in Table 1. This result compares favourably with other literature values [8,9] and was used in all subsequent $K_i$ determinations.

A typical plot for the graphical determination of $K_i$ is shown in Fig. 2. Data points in this plot are taken from the progress curve associated with the inhibition of cholinesterase by quinine. Deviations from absolute linearity were more apparent in inhibition plots when compared to (uninhibited) $K_m$ plots. In order to maximize linearity three practical precautions should be observed.

a) Enthalpograms should be corrected for any heat losses occurring during the measurement period. This can be achieved empirically by the arithmetic addition of the slope of the post-reaction baseline to the progress curve after 0.5 $t$, where $t$ is the measurement period for the entire curve. Failure to do this will

Table 1

The enthalpimetric determination of $K_m$ for the cholinesterase-catalyzed hydrolysis of butyrylcholine. Results calculated by extrapolation of the integrated Michaelis– Menten plot

| $K_m$, mmol dm$^{-3}$: | 1.85 | 1.88 | 1.61 | 1.71 | 1.68 | 1.55 | 1.57 |
|---|---|---|---|---|---|---|---|
| Mean value: | 1.70±0.13 mmol dm$^{-3}$ | | | | | | |

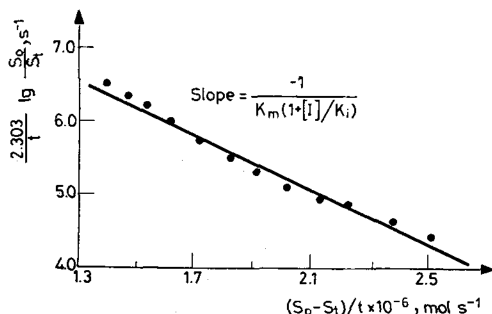


Fig. 2. Typical integrated Michaelis– Menten plot. Quinine inhibition

result in spurious $K_i$ assignments since heat loss is manifested as an apparent decrease in reaction rate.

b) Data for the plot should be extracted from the second half of the enthalpogram. In the initial stages of the reaction $S_t > K_m$ and accordingly $d^2S_t/dt^2 \simeq 0$ as evidenced by the linearity in the early portion of the enthalpogram. Typical enthalpograms are shown in Fig. 1.

c) Inhibitor concentration should be numerically close to the predicted value of $K_i$. High inhibitor concentration lengthens the time base of the enthalpogram and leads to increased heat loss.

Table 2

Comparison of $K_i$ data obtained by the integrated Michaelis—Menten and Lineweaver—Burk plots

| Inhibitor | Integrated Michaelis—Menten plot $K_i$, mol dm$^{-3}$ | LB plot $K_i$, mol dm$^{-3}$ | $I_{50}$, mol dm$^{-3}$ |
|---|---|---|---|
| Morphine | $6.3 \times 10^{-4}$ | $6.27 \times 10^{-4}$ | $7.5 \times 10^{-4}$ [10] |
| Quinine | $1.4 \times 10^{-5}$ | $1.35 \times 10^{-5}$ | |
| Procaine | $8.4 \times 10^{-5}$ | $8.03 \times 10^{-5}$ | $2 \times 10^{-4}$ [11, 12] |

$K_i$ assignments made using the recommended procedure are shown in Table 2; the results represent the mean of duplicate measurements made at a single inhibitor concentration. Assignments made using Lineweaver-Burk plots are included in Table 2 for comparison. A literature search failed to produce any correlation data for $K_i$. $I_{50}$ values have been reported for morphine and procaine inhibition and these are also presented in Table 2.

The $I_{50}$ value is an empirically defined parameter, representing the concentration of inhibitor required to decrease the enzyme activity by 50%. Although $I_{50}$ values are not fundamental parameters and are by no means synonymous with $K_i$, they do approach $K_i$ under certain conditions. This can be seen by inspection of the rate equation for a competitively inhibited enzyme reaction (obtained by differentiation of Eq. 3),

$$-\,dS_t/dt = \frac{V_{max} S_t}{S_t + K_m(1 + [I]/K_i)}.$$

Clearly at small values of $S_t$, the rate of the reaction will be halved when $[I] = K_i$.

The data compares favorably with the result obtained by the double reciprocal plot. Direct comparison of $K_i$ with $I_{50}$ is dubious for reasons mentioned above; however, the similarity between $K_i$ and $I_{50}$ figures does reduce the possibility of any gross inaccuracies in the data.

The data does illustrate the potential of enthalpimetry as a universal method for the determination of $K_m$, $K_i$, and $V_{max}$ in two experiments. Qualitative informa-

tion regarding the type of inhibition is also available from the change in slope or ordinal intercept in the same number of experiments. Lineweaver-Burk plots would require a minimum of six experiments to obtain the same data. Perhaps the most important advantage for fundamental studies is the fact that kinetic parameters need not be affected by the presence of a secondary reagent necessary with many instrumental approaches to produce a measurable species e.g. a chromophore. In many cases, the primary enzymatic event can be monitored enthalpimetrically.

The manual extrapolation procedure used to obtain data from enthalpimetric progress curves does detract from the convenience of the technique in that it is time-consuming. Work is currently in progress to allow microcomputer controlled acquisition and treatment of enthalpimetric data. Once completed, $K_m$ and $K_i$ assignments could be made in several minutes following the completion of the experiment.

The inhibition of a serum enzyme by alkaloids of pharmaceutical importance clearly has physiological consequences. However, caution is necessary when attempting to correlate in vitro data (where the substrate concentration is known and usually relatively large) with in vivo data (where little, if anything, is known about the substrate concentration, and it is likely to be exceedingly small).

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Résumé — On décrit une méthode pour déterminer les constantes d'inhibition, qui repose sur un procédé publié auparavant où $K_m$ est obtenu à partir de l'équation de Michaelis—Menten intégrée. On a choisi comme modèle l'hydrolyse de la butyrylcholine catalysée par la cholinestérase et inhibée par la morphine, la quinine et la procaïne. Les résultats peuvent être comparés favorablement aux données obtenues par Lineweaver—Burk.

ZUSAMMENFASSUNG — Eine Methode zur enthalpimetrischen Bestimmung von Hemmungskonstanten wird beschrieben, welche auf einem früher bekanntgegebenen Verfahren zur Bestimmung von $K_m$ unter Anwendung der integrierten Michaelis—Menten Gleichung beruht. Die Hemmung der durch Cholinesterase katalysierten Hydrolyse von Butyrylcholin durch Morphin, Chinin und Procain wird als Modell gewählt. Die Ergebnisse sind in guter Übereinstimmung mit Lineweaver—Burk Angaben.

Резюме. — Описан метод энтальпиметрического определения констант торможения реакцийна основе ранее сообщенчого метода определения $K_m$ при использованни интегрального уравнения Михаэлиса—Ментена. В качестве модели выбрана реакция холиностеразкатализируемого гидролиза бутирилхолина морфином, хинином и прокаином. Результаты хорошо согласуются с данными Лайнвивер—Барка.