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ENTHALPIMETRIC MEASUREMENTS OF THE STRENGTHS OF IMMOBILIZED CHOLINESTERASE INHIBITORS

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ABSTRACT

A rapid, reproducible method is described for the enthalpimetric determination of the relative inhibitory strengths of a series of 29 reversible acetylcholinesterase and butyrylcholinesterase inhibitors. The technique continuously monitors the activity of glass-immobilized cholinesterases, which can be rapidly and quantitatively reactivated simply by removal of the inhibitor. Complete inhibition curves are presented for some of the most potent inhibitors. For the strongest inhibitors, it is possible to detect amounts as low 5×10^{-9} mol.

INTRODUCTION

Cholinesterases have been amongst the most studied of enzymes, because of their vital role in the nervous systems of all animals. Two important enzymes in this class which have been recognized (E.C. 3.1.1.7. and E.C. 3.1.1.8.) are usually named after their optimum substrates, acetylcholine esterase (Ac.Ch.E) and butyrylcholine esterase (Bu.Ch.E.)

Many irreversible inhibitors of cholinesterase enzymes (such as organophosphorous nerve gases and pesticides) are well known. However, cholinesterases are also inhibited reversibly and competitively by many other compounds, usually containing quaternary nitrogen atoms, nitrogen atoms capable of acquiring a positive charge by protonation, or ester groups [1]. Such molecules often compete with substrate for binding to the enzyme. Indeed, some alkaloids, such as eserine, owe their pharmacological activity to an inhibitory mechanism [2].

Experimentally, it has been shown that drugs effective against Parkinsonism (including Parkinson's disease and similar nervous disorders) inhibit Bu.Ch.E. more strongly than Ac.Ch.E. Conversely, powerful neuromuscular blocking agents inhibit Ac.Ch.E. more effectively than Bu.Ch.E. [3]. Consequently, any method which offers a rapid and repeatable determination of the extent of inhibition of these enzymes may, in addition to

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providing a rapid assay of "total inhibitory strength" of an unknown solution, also provide a useful screen for the initial testing of new drugs. Such a system should, ideally, conform to the following requirements.

(a) General detection system for the determination of enzyme activity

A great number of cholinesterase assay procedures have been described, based on the measurement of acetic acid, or other moiety released during hydrolysis, or on the direct measurement of acetylcholine remaining after hydrolysis [1]. However, when dealing with unknown solutions, or a wide variety of inhibitors, interferences using these detection systems may occur. For example, acidic or basic inhibitors may give anomalous results, and inhibitors similar in structure to acetylcholine could interfere with acetylcholine measurement techniques. One universal reference (after calibration) of the extent of reaction is the measurement of an enthalpy change. In the case of a limited amount of enzyme exposed to an excess of substrate, the enthalpy change per unit time is exactly proportional to activity of the enzyme present, and any decrease in this rate of heat output upon addition of an inhibitor is in direct proportion to the amount of inhibition occurring. The advantages of such detection systems have previously been recognized, and calorimetric measurements of enzyme activity have been used to determine irreversible cholinesterase inhibitors, such as organophosphorous pesticides [4,5] and also reversible inhibitors [6].

(b) Precise temperature control

Precise measurements of enzyme activity are plagued by the problems presented by temperature control. Absolute reaction rates of enzyme reactions are temperature-dependent with changes of only fractions of degrees centigrade producing significant alterations in rate. As there are enthalpy changes associated with all reactions, precise control of temperature can present serious difficulties. The calorimetric detection device used in this work was of the isothermal, or "heat-leak" type, designed to eliminate any temperature changes, thereby totally eliminating this problem [7].

(c) Maintenance of uninhibited enzyme activity and rapid assay of inhibitors

Use of immobilized enzymes will ensure maintenance of uninhibited enzyme activity and enable rapid determination of inhibitory power, provided all inhibitors used are reversible. However, despite these and other advantages of immobilized enzymes little work of this type appears to have been conducted.

This paper describes a rapid, reproducible calorimetric technique capable of assessing the relative inhibitory strengths of a wide variety of reversible

inhibitors of cholinesterase enzymes. Additionally, such a system also allows a measurement of the "total inhibitory strength" of an unknown solution, free from possible interferences associated with the presence of unknown or uncharacterized compounds. For particularly effective inhibitors, quantities as low as 5×10^{-9} mol can be detected.

EXPERIMENTAL

Reagents

Acetylcholinesterase (Type III, electric eel), butyrylcholinesterase (Type IV-S, horse serum), all inhibitors and substrates were obtained from Sigma Chemical Co. (U.K.). Trishydroxymethylaminomethane (THAM), controlled porosity glass, aminopropyltriethoxysilane (APTES) and all other chemicals were purchased from BDH Ltd. (U.K.). Where advised, compounds were stored desiccated at 4°C.

Methods

Acetylcholinesterase (1.36 mg, 500 units) and butyrylcholinesterase (20 mg, 426 units) were each covalently coupled to glutaraldehyde-activated alkylamine glass (0.5 g, 13.3 $m^2\ g^{-1}$, 85 Å mean pore diameter) by a previously described procedure [9].

The flow microcalorimeter used was an LKB 10700-1 model, operated in the continuous flow mode [7]. Glass-immobilized enzyme was loosely packed into the flow cell using (LKB) teflon filter papers situated at the column inlet and outlet. Output from the microcalorimeter was amplified by a Keithley 150B Null-voltmeter, to a level suitable for display on a 100 mV chart recorder. Throughout the experiments described the temperature was maintained at 25.0 ± 0.01°C. Under these conditions the sensitivity of heat measurements was $< 1\ \mu W$.

Determinations were initiated by pumping through a buffer solution (0.1 M THAM, pH 7.8) until thermal equilibrium, as evidenced by a horizontal baseline, was obtained. Buffer and substrate (50×10^{-3} M) were then pumped over the immobilized enzyme, until another steady state (horizontal trace) was reached. Saturation of the enzyme was demonstrated by increasing the substrate concentration still further, and observing no increase in heat output. Under these conditions, the rate of heat output was dependent only on the apparent activity of the immobilized enzyme, and the overall enthalpy of the enzyme-catalysed reaction. Fresh solutions were prepared every 3 to 4 h, due to slow non-enzymatic hydrolysis of the substrate. Deionized water was used throughout the study.

Standard solutions of each inhibitor were prepared in the buffer/substrate

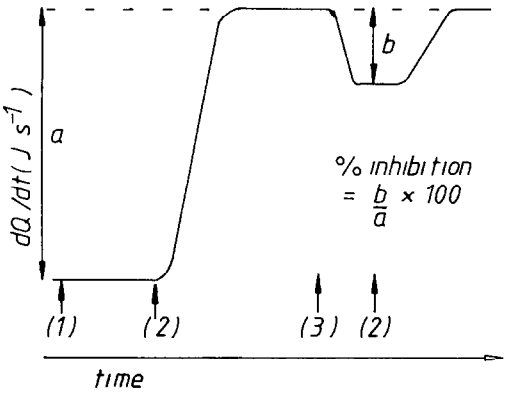

Fig. 1. Cycle of operations and instrument response for the determination of reversible inhibitors of immobilized cholinesterase. Injection of buffer (1), buffer + substrate (2), buffer + substrate + inhibitor (3).

solution, and pumped through the immobilized enzyme reaction cell in the usual way, until another steady state was obtained, reflecting the new (reduced) level of enzyme activity. A schematic representation of a typical trace is illustrated in Fig. 1, together with a simple formula for calculating percentage inhibitions. Finally, the reversible inhibitor was washed off, by passing buffer and substrate over the immobilized enzyme, thereby regenerating full enzyme activity, prior to determining another inhibitor.

RESULTS AND DISCUSSION

The percentage inhibitions of Ac.Ch.E and Bu.Ch.E. produced by 10^{-2} M (ca. 5 cm^3) of a variety of inhibitors are shown in Table 1, together with the percentage inhibitions produced by 10^{-3} M solutions of some of the stronger inhibitors.

Although many compounds have been reported in the literature to inhibit the cholinesterase system, quantitative data are scarce. The results that are quoted are difficult to compare because of the different methods and conditions used to determine inhibition. Most investigations classify inhibitors simply as strong, weak or non-inhibitors. The probable reason for the lack of comparable, quantitative data in the literature for the reversible inhibitors of the cholinesterase system is due either to the length of time taken (including equilibration time) for one run, difficulty in using exactly the same activity of enzyme per run, and the expense of using soluble enzymes, or a combination of all three.

The present method, although apparently slightly less sensitive than those using soluble enzymes, circumvents all these difficulties, and should therefore be of considerable interest in the determination of relative inhibitor strengths.

TABLE 1

Percent inhibition of immobilized Ac.Ch.E. and Bu.Ch.E by reversible inhibitors [a]

Inhibitor	Imm.Ac.Ch.E.		Imm.Bu.Ch.E.	
	[b]	[c]	[b]	[c]
Antazoline phosphate	24	< 5	100	66
Antipyrene	5		58	
Arecoline. HCl	5		14	
Atropine sulphate	8		41	
Atropine methyl nitrate	5		42	
Caffeine	53	20	10	5
Diphenhydramine. HCl	13	< 5	92	37
Eserine sulphate	100	100	100	100
Fluoride (F^-)	46		71	
Histamine. HCl	< 5		13	
Methylene Blue	100		98	
Naphazoline. HCl	29	< 5	100	72
Nicotine sulphate	11		32	
Phenazine methosulphate	95		100	85
Pilocarpine nitrate	36		25	
Procaine. HCl	16		83	
Pyridoxal. HCl	25		21	
Pyridine-2-aldoxine methiodide	23		42	
Quinacrine. HCl	64		100	69
Quinine sulphate	14	< 5	97	51
(−)-Scopolamine. HBr	< 5		17	
L(−)-Sparteine	23	< 5	93	27
Thiamine. HCl	32		25	
Tolazoline. HCl	42		96	44
Tetramethyl ammonium bromide	< 5		< 5	
Tetraethyl ammonium bromide	< 5		< 5	
Trimethyl phenyl ammonium bromide	27		23	
Tetra (*n*-propyl) ammonium bromide	48		34	
Tetra (*n*-butyl) ammonium bromide	24		46	

[a] Ac.Ch.E and Bu.Ch.E denote acetyl or butyrylcholinesterase; THAM buffer (0.1 M, pH 7.8; $T = 25°C$).

[b] 10^{-2} or 10^{-3} M inhibitor.

[c] 10^{-3} M inhibitor.

A limited study of the effect of alkaloids on horse serum butyrylcholinesterase has been carried out, using soluble enzymes and a thermistor as heat sensor, by Grime and Tan [6], to study the inhibition of eserine, quinine, procaine, atropine, morphine, codeine, pilocarpine, thiamine and caffeine at varying concentrations. In this work, 10^{-2} M was the concentration most used. Codeine was unobtainable and morphine was not soluble enough in buffer to give a 10^{-2} M solution. A comparison of results with those of the present study is given in Table 2. Grime and Tan [6] quote the inhibitory power of the alkaloids as eserine ≫ quinine > procaine > atropine > thiamine > pilocarpine > caffeine. This trend is reflected in this work,

TABLE 2

Percent inhibition of soluble [a] and immobilized [b] Bu.Ch.E by some inhibitors

Inhibitor	Sol.Bu.Ch.E [c]	Imm.Bu.Ch.E
Eserine	100	100
Quinine	100	97
Procaine	95	83
Atropine	70	41
Thiamine	65	25
Pilocarpine	55	24
Caffeine	55	10

[a] Grime and Tan[6]; THAM buffer (0.1 M, pH 8); 10^{-2} M inhibitor.
[b] This work; THAM buffer (0.1 M, pH 7.8) 10^{-2} M inhibitor.
[c] Bu.Ch.E denotes butyrylcholinesterase (E.C. 3.1.1.8).

thus justifying this method as useful for the comparison of inhibitory power.

A further possible application of this technique is in the determination of "total inhibitor strength" of an unknown solution from calibration curves. A plot of the percent inhibition of immobilized Bu.Ch.E. by eserine, phenazine or naphazoline is shown as an example in Fig. 2.

Although the majority of these inhibition studies were conducted at pH 7.8 in 0.1 M THAM, a brief study of inhibitors at higher (8.5) and lower (7.0) pH values was conducted, to determine the effects (if any) of small deviations in pH on inhibitory power. Working at either of these pH values did present problems. There was a rapid non-enzymatic hydrolysis of substrate at pH 8.5, necessitating fresh solutions of buffer to be made hourly. At pH 7.0 problems arose due to the low buffer capacity of THAM. Use of an

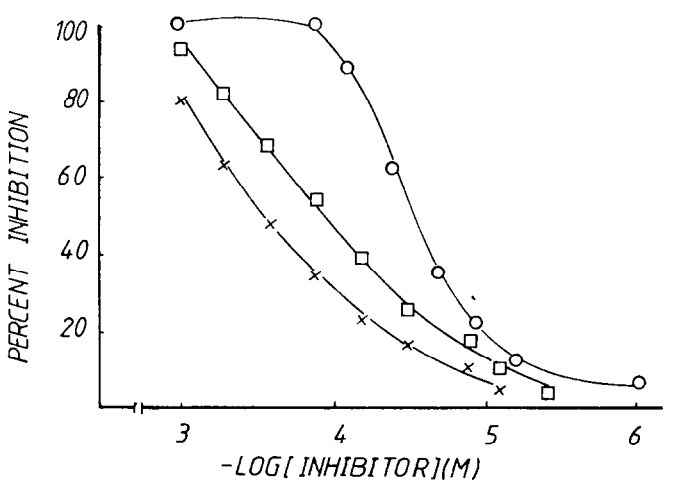

Fig. 2. Inhibition of immobilized butyrylcholinesterase by eserine (○), phenazine methosulphate (□) or naphazoline (×) in THAM buffer (0.1 M, pH 7.8).

TABLE 3

Percent inhibition of immobilized Ac.Ch.E. and Bu.Ch.E. by diphenhydramine and flouride as a function of pH [a]

Enzyme	Imm.Ac.Ch.E			Imm.Bu.Ch.E		
	pH 7.0	7.8	8.0	7.0	7.8	8.0
Diphenhydramine [b]	18	13	11	100	92	65
Diphenhydramine [c]	–	–	–	74	37	17
Flouride [b]	93	42	18	100	71	21

[a] Conditions: 0.1 M THAM buffer; Ac.Ch.E denotes acetylcholinesterase.
[b] 10^{-2} M inhibitor.
[c] 10^{-3} M inhibitor.

oxygen-base buffer at pH 7.0 (e.g., phosphate) offered no advantages. Although possessing large buffer capacities around pH 7.0, their enthalpies of protonation were typically one order of magnitude lower than that of THAM (4.8 vs. 47.5 kJ mol^{-1}) [8] resulting in a decrease in heat output.

Studies were also carried out in 250 mM imidazole buffer at pH 7.0. Again the signal given was less than with THAM, probably due to its lower enthalpy of protonation.

In most cases, little or no difference in inhibitory power appeared to occur as a function of pH. However, in the case of two types of compounds, inhibitory power appeared to be dramatically affected by pH. Table 3 illustrates the variation of inhibitory power of cholinesterase inhibitors with pH, for (a) diphenhydramine (typical of compounds with structures R-NMe_2, containing a basic nitrogen but no positive charge) and (b) the fluoride ion. It can be seen that, at pH 7.0, both are strong inhibitors of (particularly) Bu.Ch.E. Indeed, 5 cm^3 of each solution (10^{-5} M) are readily detectable at pH 7.0. However, at pH 8.5 inhibition of both is less strong.

It seems that increased acidity enhances the inhibitory strength of these species. Hence, when comparing inhibitor strengths of compounds of these types, the likely effect of pH on inhibition properties should be considered.

Neither NMe_4^+ nor NEt_4^+ inhibit either enzyme noticeably, whereas $N(n\text{-Pr})_4^+$ and $N(n\text{-Bu})_4^+$ inhibit both fairly strongly (Table 1). This was surprising as NMe_4^+ most resembles the choline part of acetyl or butyrylcholine. The observation that increasing the chain lengths of the alkyl groups (up to tetra *n*-propyl for Ac.Ch.E. or up to tetra *n*-butyl for Bu.Ch.E.) in the tetra-substituted ammonium ions, or that replacement of one methyl group in the tetramethyl ammonium ion by one phenyl group, led to an increase in inhibition in both cases, is evidence that hydrophobic interactions play an important part in enzyme binding of inhibitors [10].

In order to react at the enzyme-active site, a potential substrate molecule must be "held" by the electrostatic attraction between an anionic site and a positively charged atom (nitrogen) whilst its ester group is attached to the

esteratic site. Thus, in principle, groups which can block either of these sites will act as inhibitors. As most of the inhibitors studied have either a positive or a basic nitrogen atom, these should, in principle, be capable of inhibiting cholinesterases, the strength of inhibition presumably being determined by the strength of attraction (i.e., the "fit") between the positive centre in the inhibitor and the anionic site.

In conclusion, the rapidity and constancy of uninhibited enzyme activity, and its ease of regeneration make this technique of considerable interest in the study of reversible inhibitors. The absolute sensitivity, although apparently slightly lower than in corresponding studies using soluble enzymes, is still sufficient to detect 5×10^{-9} mol of the strongest inhibitors. The quantitative comparison of individual inhibitor strengths and the combination of the very general thermal detection in conjunction with immobilized enzymes appears ideally suited to measurements of "total inhibitory strengths" of unknown solutions, where unexpected individual components may interfere with the detection system.

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