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A SIMPLE AND SENSITIVE METHOD FOR THE DETERMINATION OF ENZYMATIC HYDROLYSIS OF VARIOUS ESTERS

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SUMMARY

A simple method was developed to determine the enzymatic hydrolysis of various esters. The principle of this method is that $^{14}CO_2$ produced from interaction between $NaH^{14}CO_3$ and acid produced from esters hydrolyzed by esterases is trapped with 2-phenethylamine and counted with a liquid scintillation counter. The advantages of this method are manyfold: (a) it is much simpler and more sensitive than the Warburg technique, allowing the use of substrate concentrations as low as 10^{-4} M acetylcholine; (b) it is applicable to any ester substrates, in contrast to spectrophotometric methods which are confined to thioesters as substrates; (c) it is much less expensive than the radioactive substrates used in the radioisotopic method; and finally, (d) it is much less complex in operation than the pH-stat method.

INTRODUCTION

There are four major techniques available for studying enzymatic hydrolysis of esters: Warburg manometric[1,2], spectrophotometric[3], radioisotopic[4–6], and pH static[7,8] methods. However, none of these techniques satisfy all of the following important criteria.

(a) Sensitivity

The sensitivity of the Warburg manometric method is the poorest of all in detecting product formation from esters. For this reason, it does not allow the use of substrate concentrations lower than 10^{-3} M acetylcholine[1,2].

(b) Substrates

No substrate other than thioesters can be used in spectrophotometry. This limits the applicability of this method with regard to the determination of hydrolysis of various non-thioesters, and it is used only for the determination of esterase activity[3]. Although the radioisotopic method allows the use of various substrates, it is expensive

and difficult to obtain these radioactive substances. Thus, its use is again limited to the determination of esterase activities rather than substrate specificities[4-6].

(c) Simplicity

Theoretically, the pH-stat is an ideal method for the determination of both enzyme activities and substrate specificities. However, the complexity of the procedure is a major disadvantage which prevents wide use of this technique[7,8]. For example, in order to raise the sensitivity of this technique, the concentration of NaOH used to neutralize the acid formed from ester hydrolysis has to be kept as low as possible. However, low concentrations of NaOH tend to consume large volumes of NaOH solution, which dilutes and changes the substrate concentrations in the reaction medium. Thus, the concentration of NaOH solution has to be adjusted to the optimal in the system for a particular concentration of substrate determined. Every change of the substrate concentration requires cleaning and drying of the whole system, together with a change of the NaOH solution.

The radioactive bicarbonate method developed in this research allowed us to study any esters as substrates hydrolyzed by any esterases and to use concentrations of acetylcholine as low as $1 \cdot 10^{-4}$ M. The procedure is simple to operate and the materials needed are inexpensive to obtain.

MATERIALS AND METHODS

Butyrylcholinesterase used in this study was prepared from horse serum with an enzymatic activity of 6 units/mg of protein (Worthington Biochemical Corp., Freehold, N.J.). Substrates studied included acetylcholine iodide, and butyrylcholine iodide. The rates of hydrolysis of some enzyme inhibitors were also studied, including physostigmine sulfate, neostigmine methylsulfate, diiospropyl fluorophosphate and trichlorphor. Radioactive sodium bicarbonate ($NaH^{14}CO_3$) was purchased from New England Nuclear, Boston, Mass., with specific activity of 0.25 Ci/4.3 g.

Experiments were done using a Warburg apparatus with 0.4 ml of 2-phenethylamine placed in the central well of the flask to trap $^{14}CO_2$ produced. The main compartment of the 15-ml Warburg flask contained 2.0 ml of modified Krebs–Ringer bicarbonate solution with 1/10 the normal concentration of $NaH^{14}CO_3$ and a radioactivity level of $2.22 \cdot 10^5$ dpm ($2.3 \cdot 10^{-3}$ M $NaH^{14}CO_3$, $7.5 \cdot 10^{-2}$ M KCl, $7.5 \cdot 10^{-2}$ M NaCl, $4 \cdot 10^{-2}$ M $MgCl_2$). Butyrylcholinesterase was added to the main compartment of the flask in vol. of 50 μl with final concentration of 1.25 units/ml. The side arm contained 0.5 ml of substrate dissolved in the modified Kreb's solution. The air in the flask was replaced with N_2. The Warburg flasks were shaken for 15 min before the substrate was dumped into the main compartment. The reaction mixture was shaken for various periods of time and the 2-phenethylamine with $^{14}CO_2$ was transferred to a counting vial containing 10 ml of scintillation cocktail (2,5-diphenyloxazole, 7 g; naphthalene, 100 g; dioxane, 1000 ml). The central well was washed twice with 0.3 ml each of methanol and the washings were added to the scintillation cocktail and counted with a Beckman's Liquid Scintillation Counter.

Control experiments were run in identical conditions but without enzyme in the reaction mixture. The CO_2 produced by spontaneous hydrolysis (control experiments) was subtracted from the total CO_2 produced in flasks which contain enzymes. The net

CO_2 production due to enzymatic hydrolysis was then plotted in all figures. The highest substrate concentration used was 10^{-3} M which produced $2.50 \cdot 10^{-6}$ moles of CO_2 when the substrate was hydrolyzed completely. Since the consumption of substrate had to be less than 10% of the total substrate present in the reaction mixture in order to keep the substrate concentration constant, the CO_2 produced was less than $2.50 \cdot 10^{-7}$ moles. It is known that 0.4 ml of 2-phenethylamine is capable of trapping more than $4.25 \cdot 10^{-7}$ moles of CO_2 (ref. 9), so there is no doubt that all CO_2 produced in this system can be effectively trapped in 2-phenethylamine used.

RESULTS

Effects of temperature on $NaHCO_3$ decomposition

In order to investigate the effects of temperature on the decomposition of $NaHCO_3$, the bicarbonate solution was incubated at various temperatures without enzymes and substrates. $NaHCO_3$ was significantly decomposed at 37 °C but very slowly at 25 °C (Fig. 1). The amount of $NaHCO_3$ decomposed during 60 min incu-

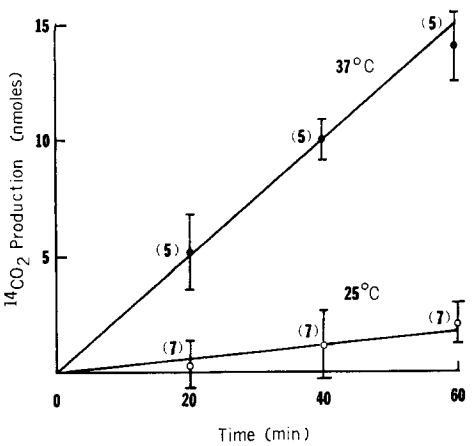

Fig. 1. Effects of various temperatures on $^{14}CO_2$ formation from $NaH^{14}CO_3$. Bars represent standard errors.

bation was 2.1% of the total in the incubation mixture at 37 °C and only 0.33% at 25 °C. Thus, the subsequent study was conducted at 25 °C throughout. Since the substrate concentration used in this study was lower than 10^{-3} M and the rate of cholinesterase hydrolysis was low at low substrate concentrations[8], a bigger ratio between the rate of enzymatic hydrolysis and that of nonenzymatic decomposition of $NaHCO_3$ could be obtained at lower temperatures than at higher ones.

Hydrolysis of cholinesters by butyrylcholinesterase

Acetylcholine at 10^{-3} M was hydrolyzed by butyrylcholinesterase at a linear rate during the 60 min period of hydrolysis studied (Fig. 2). The rates of hydrolysis were linear for the first 40 min and then slowed down when the acetylcholine concentration was 10^{-4} M. Similar results were obtained with butyrylcholine as a substrate except the rate of hydrolysis slowed down 20 min after the incubation with substrate

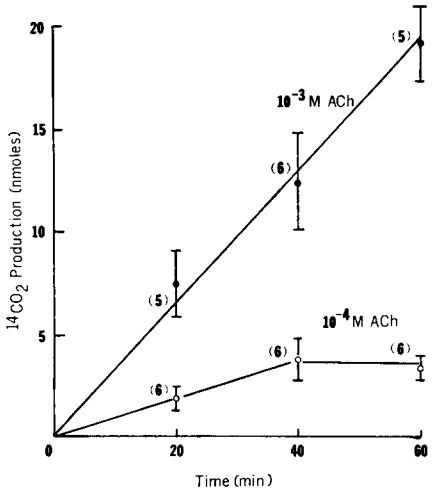

Fig. 2. Patterns of butyrylcholinesterase hydrolysis of various concentrations of acetylcholine (ACh) as a function of time. The concentration of butyrylcholinesterase was 1.25 units/ml and bars represent standard errors.

concentration of 10^{-3} M (Fig. 3). The rates of acetylcholine hydrolysis by butyrylcholinesterase were also dependent on the concentrations of butyrylcholinesterase within the ranges studies (Fig. 4).

Hydrolysis of enzyme inhibitors by butyrylcholinesterase

Attempts had been made to determine the rate of hydrolysis of enzyme inhibitors, including neostigmine, physostigmine, diisopropyl fluorophosphate and tri-

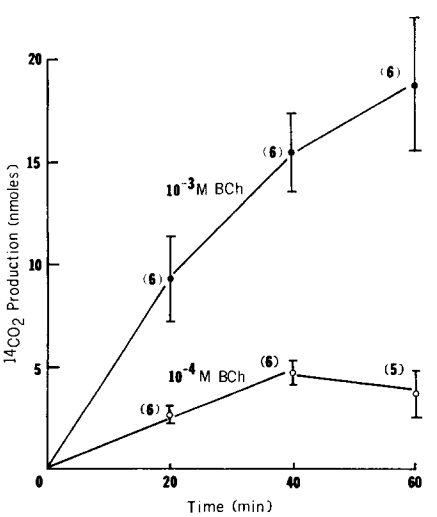

Fig. 3. Patterns of butyrylcholinesterase hydrolysis of various concentrations of butyrylcholine (BCh) as a function of time. The concentration of butyrylcholinesterase was 1.25 units/ml and bars represent standard errors.

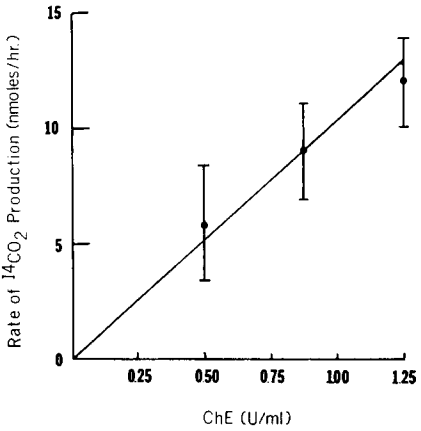

Fig. 4. Relationships between rates of hydrolysis of acetylcholine (10^{-3} M) and concentrations of butyrylcholinesterase (ChE). Bars represent standard errors with $n=6$.

chlorphor. There was no significant amount of acid products which could be detected during the 3-h period of incubation, although a considerable quantity of esters was hydrolyzed nonenzymatically (chemically) (Table I). These results support the idea that these compounds are hydrolyzed by butyrylcholinesterase slowly, if at all.

TABLE I

NONENZYMATIC HYDROLYSIS (AUTOHYDROLYSIS) OF ESTERS (CHOLINESTERASE INHIBITORS)

Esters (10^{-4} M)	*Number of expts*	*Rate of hydrolysis* (nmoles/h)*
Physostigmine	7	4.66 ± 1.06
Neostigmine	7	9.23 ± 0.68
Diisopropyl fluorophosphate	6	6.08 ± 0.90
Trichlorphor	5	3.66 ± 1.12

* Mean ±S.E.

DISCUSSION

The major criticism for the use of the Lineweaver–Burk plot is that this method relies heavily on the rate of hydrolysis obtained at low substrate concentrations where the reliability of the data is the least and the variability the greatest[10]. The bicarbonate method developed in this study is unique in that it can determine rather accurately the rates of hydrolysis of esters at low substrate concentrations. This certainly is an improvement over the Warburg manometric method[1,2]. Secondly, this method is applicable in determining enzymatic hydrolysis of esters with any changes in types of substrates or enzymes. This wide range of applicability overcomes the limitations of the spectrophotometric method which can be used only with thiocholine esters as substrates[3], and the radioisotopic method which requires specific radioactive esters as substrates[4–6]. Finally, the procedure is simple and the facilities inexpensive. These are the great advantages over the pH-stat method[7,8]. The only equipment

needed for this experiment are Warburg flasks and an incubation shaker. A Dubnoff incubation shaker (Precision Sci. Co., Chicago, Ill.), for instance, may be used instead of a Warburg apparatus.

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