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CHOLINESTERASE ACTIVITIES OF INTACT PLATELETS AS MEASURED BY AN IMPROVED RADIOMETRIC METHOD

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SUMMARY

Because of the extremely low cholinesterase activities of human platelets, an assay method more sensitive and specific than those previously available was needed. Such an assay was developed employing ^{14}C-labeled substrates. In this assay system, less than 10^{-5} unit of cholinesterase activity can be measured conveniently. Data were also obtained from a nonspecific and less sensitive colorimetric method for the purpose of comparison. In general, the results from these two assay methods seem to be in fairly good agreement. Furthermore, kinetic data also suggest that the cholinesterase activity of intact platelets is most likely an acetylcholinesterase.

INTRODUCTION

There are at least a dozen methods available for the assay of cholinesterase activities of tissue and biological fluid. A manometric[1–4] and a colorimetric method[5] each have been employed to measure the cholinesterase activities of platelets. Because of the extremely low cholinesterase activity of platelets, these two methods were found to be unsuitable for kinetic studies. A more sensitive and specific radiometric assay method, a modification of the procedure of Potter[6], was therefore developed. In this report, more detailed studies of the assay method, such as optimal substrate (acetylcholine) concentration, pH, and enzyme concentration as well as specific inhibitor studies which were not described fully before[6] were investigated. Further, the application of this method to kinetic studies of platelet cholinesterase activities was investigated.

MATERIALS AND METHODS

Human platelets were prepared essentially according to a differential centrifugation method (washed platelets) previously described[7]. The platelets were washed 4 times with a volume of citrated saline (1 part 3.2% sodium citrate + 19 parts 0.9% NaCl (pH 6.5)) which was equivalent to one-third of the volume of the original

platelet-rich plasma. Platelets isolated by gel filtration (gel platelets) were prepared by the method of Tangen *et al.*[8]. Both platelet preparations were studied for erythrocyte contamination by use of phase contrast microscopy and for plasma protein contamination by estimation of butyrylcholinesterase activity (butyrylcholine used as substrate) in the final wash or suspension fluid. Similar cholinesterase activities were measured using platelet preparations made by both methods, although the gel platelets consistently appeared to have less plasma protein contamination (unpublished results) than did the washed platelets.

Several significant changes were made in the original radiometric assay method[6]. (1) [$^{14}C_1$]Butyrylcholine was used instead of benzoylcholine in addition to [$^{14}C_1$]acetylcholine and [$^{14}C_1$]acetyl-β-methylcholine as substrates (all were products of New England Nuclear). Since butyrylcholine is known to be a better substrate than acetylcholine for plasma butyrylcholinesterase, it was therefore chosen as a substrate to check for possible butyrylcholinesterase contamination in platelet preparations. (2) The substrate concentration (*e.g.* acetylcholine) was reduced to $2.7 \cdot 10^{-4}$ M (final concn; with approx. 20 000 cpm) in order to increase the sensitivity of the measurement and to minimize possible substrate (acetylcholine) inhibition (Fig. 1). (3) The pH was also changed to 8.0 in 0.05 M Tris to insure maximum enzyme activity (Fig. 2). (4) $MgCl_2$ was omitted from the reaction mixture, since it was found to have no effect on enzymatic activity (Table I). Siliconized clotting tubes (10 mm $\times$ 75 mm, Kimble Glass Co.) were used to store the dried substrates (under P_2O_5 *in vacuo*) and for subsequent enzymatic assay. Normally, a final reaction mixture of 0.1 ml contained: 5 μmoles Tris (pH 8.0); 0.027 μmole substrate (or otherwise as indicated); 15 μmoles NaCl (to maintain isotonicity); and $2 \cdot 10^8$–$2.5 \cdot 10^8$ platelets. After incubation at 37 °C for 10–60 min, the enzymatic reaction was stopped by the addition of 0.1 ml 0.2 M HCl. The $^{14}C_1$-labeled acetic acid or butyric acid was extracted with 2 ml of toluene–isoamyl alcohol (5:1, v/v) by vigorous mixing on a vortex mixer for 1–2 min. The organic phase was separated by brief centrifugation (Adams Serofuge, 1000 $\times$ *g*, 5 min) and 1 ml of aliquot was transferred to a scintillation vial containing 4 ml of absolute ethanol and 10 ml of 1% phenylbiphenyloxadiapole-1,3,4 in toluene. Samples were counted in a Nuclear Chicago scintillation counter. The extraction efficiency was found to be 70% for [^{14}C]acetic acid and was not dependent upon the acetate concentration in the reaction mixture; the extraction efficiency of [^{14}C]butyric acid was found to be 93%. The counting efficiency was found to be 75% and no quenching was seen when platelets were used as the enzyme source. The enzymatic activity (v) is expressed as μmoles of substrate hydrolyzed (at particular substrate concentration) per min at 37 °C and pH 8.0 or as units (1 unit is defined as that amount of enzyme that will catalyze the hydrolysis of 1 μmole acetylcholine per min). The data were calculated based on an internal standard (acetylcholine, butyrylcholine or acetyl-β-methylcholine) and the extraction and counting efficiency were also corrected. Among the three substrates tested the rate of hydrolysis of acetylcholine by human intact platelets was found to be 10–20 times higher than butyrylcholine and acetyl-β-methylcholine (see Table I) and consequently acetylcholine was chosen as the substrate for routine assay and kinetic studies. In this assay system, a cholinesterase activity as low as 10^{-4}–10^{-5} unit can be measured conveniently. Choline acetyltransferase activity was not detected in platelets by the procedure of Fonnum[9].

RESULTS AND DISCUSSION

Kinetic data concerning the cholinesterase activity of intact human platelets as obtained by the modified radiometric assay method are shown in Figs 1–4. It

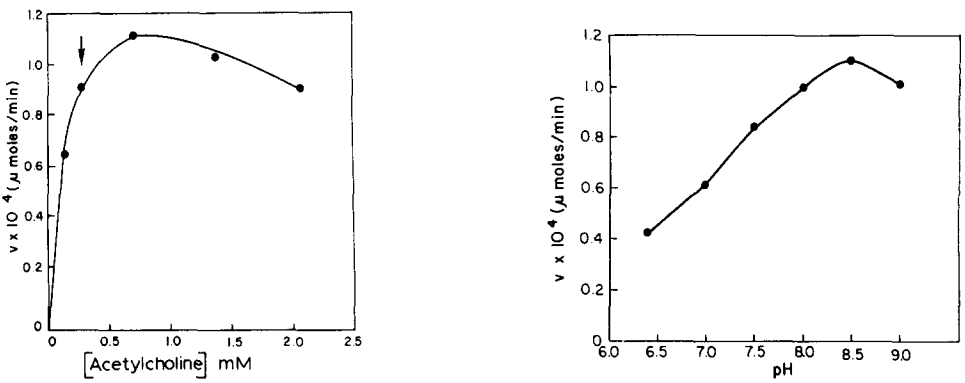

Fig. 1. Cholinesterase activities of intact platelets: Effect of [$^{14}C_1$]acetylcholine concentrations on enzymatic activity. The assay mixture of 0.1 ml contained: 5 μmoles Tris buffer (pH 8.0); 15 μmoles NaCl; [$^{14}C_1$]acetylcholine, as indicated; and $2 \cdot 10^8$ gel platelets. After incubation at 37 °C for 20 min, the labeled acetate was analyzed as described in the text. Similar data have been obtained with washed platelets. K_m estimated from a Lineweaver–Burk plot was found to be at $1.5 \cdot 10^{-4}$ M. The arrow indicates the acetylcholine concentration ($2.7 \cdot 10^{-4}$ M) used for routine assay.

Fig. 2. Cholinesterase activity of intact platelets: Effect of pH on the enzymatic activity. The assay mixture of 0.1 ml contained: 5 μmoles Tris buffer (pH as indicated); 15 μmoles NaCl; 0.027 μmole [$^{14}C_1$]acetylcholine; and $2 \cdot 10^8$ gel platelets. After incubation at 37 °C for 30 min, the labeled acetate was analyzed as described in the text.

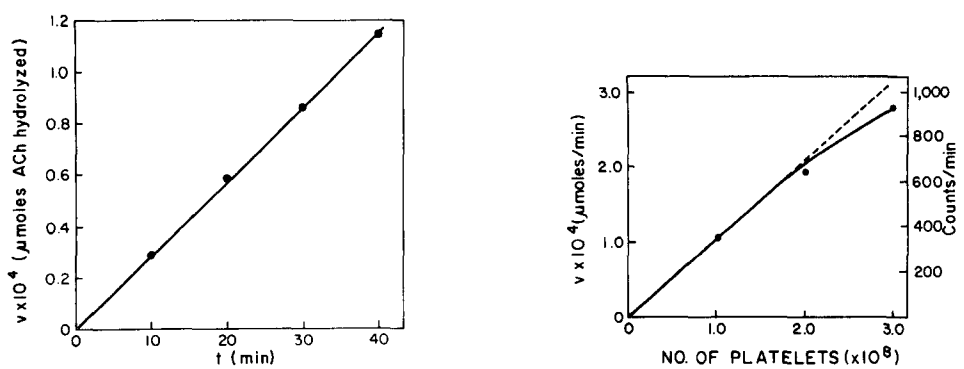

Fig. 3. Cholinesterase activity of intact platelets: Effect of incubation time on the enzymatic activity. The assay mixture of 0.1 ml contained: 5 μmoles Tris buffer (pH 8.0); 15 μmoles NaCl; 0.027 μmole [$^{14}C_1$]acetylcholine; and $2 \cdot 10^8$ washed platelets. After incubation at 37 °C for the period of time indicated, the labeled acetate was analyzed as described in the text.

Fig. 4. Cholinesterase activity of intact platelets: Effect of platelet concentration on enzymatic activity. The assay mixture of 0.1 ml contained: 5 μmoles Tris buffer (pH 8.0); 15 μmoles NaCl; 0.027 μmole [$^{14}C_1$]acetylcholine; and gel platelets as indicated. Other assay conditions were same as described in the text.

can be seen in Fig. 1 that the cholinesterase activity is closely related to the substrate (acetylcholine) concentration. The typical acetylcholine inhibition at higher concentration (greater than 1 mM) may also indicate that the human platelet enzyme is likely an acetylcholinesterase[10]. The apparent observed K_m of acetylcholine was determined to be $1.5 \cdot 10^{-4}$ M from a Lineweaver–Burk plot of the data shown in Fig. 1. The pH profile (Fig. 2) indicates that the optimal pH seems to be at 8.5; however, it was found that at that pH the nonenzymic hydrolysis of substrates (acetylcholine, butyrylcholine and acetyl-β-methylcholine) were exceptionally high (*e.g.* the spontaneous hydrolysis of acetylcholine at pH 8.5 ($0.3073 \cdot 10^{-4}$ μmole/min at 37 °C) was found to be 3 times higher than at pH 8.0 ($0.1016 \cdot 10^{-4}$ μmole/minn)) and consequently pH 8.0 was chosen for routine measurements. The enzymatic activities were also found to be similar in both phosphate and Tris buffers.

It can be seen in Fig. 3 that the reaction rate was linear with time. However, it should be pointed out that since the substrate concentration for the routine assay is not at the saturation level, it is important that the incubation time for each determination be limited to an interval during which less than 10% of the substrate is hydrolyzed in order to assure that the true initial velocity is measured. In addition, because of the substrate (acetylcholine) inhibition, V of acetylcholine can only be estimated from a double reciprocal plot; V obtained from the extrapolated value was generally found to be 1.5–1.7 times higher than the rate of acetylcholine at $2.7 \cdot 10^{-4}$ M.

A slight nonlinearity of reaction rate at higher platelet concentrations was noted (Fig. 4). Further studies indicated that this nonlinearity was most probably caused by the product inhibition (see Table II; K_i of choline was estimated to be approx. $6 \cdot 10^{-4}$ M), since choline accumulates in the reaction mixture as the enzymatic reaction proceeds. Choline is known to be an inhibitor of cholinesterase in other systems[10]. Additional possibilities, such as substrate exhaustion, further metabolism of acetate by platelets, or the possible presence of inhibitor(s) of the enzyme in the platelet suspension have been eliminated by control experiments.

Additional information concerning other properties of the platelets cholinesterase activities was obtained by the radiometric procedure and this is shown in Tables I and II. It can be seen in Table I that platelets hydrolyze acetylcholine at a rate much greater than butyrylcholine and acetyl-β-methylcholine. In addition, the butyrylcholine activity of gel platelets was also found to be less than that of washed

TABLE I

SUBSTRATE SPECIFICITY OF CHOLINESTERASE FROM INTACT HUMAN PLATELETS

Data were obtained by radiometric method using [$^{14}C_1$]acetylcholine, [$^{14}C_1$]butyrylcholine and [$^{14}C_1$]acetyl-β-methylcholine (all final concentration at $2.7 \cdot 10^{-4}$ M) as substrates. Other assay conditions are as described in the text. Numbers in parentheses indicate the number of determinations.

Methods of preparation	*Activity ($\times 10^{-4}$ μmole/min)/$2 \cdot 10^8$ platelets*			*a*/*b*	*a*/*c*
	Acetylcholine (a)	*Butyrylcholine (b)*	*Acetyl-β-methylcholine (c)*		
Washed platelets	0.780 ± 0.369 (12)	0.125 ± 0.075 (9)	0.067 ± 0.034 (7)	6.2	11.6
Gel platelets	1.106 ± 0.420 (16)	0.061 ± 0.017 (7)	0.086 ± 0.036 (9)	18.1	12.8

TABLE II

EFFECT OF SUBSTRATES, PRODUCTS, MONO- AND DIVALENT IONS, AND INHIBITORS ON THE CHOLINESTERASE ACTIVITIES OF INTACT HUMAN PLATELETS

Data were obtained by radiometric method using [$^{14}C_1$]acetylcholine (final concn $2.7 \cdot 10^{-4}$ M) as substrate. Other assay conditions are as described in the text.

Addition	*Final concn (M)*	*% activity of control*
None (control)		100
5,5′-Dithiobis-(2-nitrobenzoic acid)	$2 \cdot 10^{-3}$	104
Acetylthiocholine	$1 \cdot 10^{-3}$	16
Butyrylthiocholine	$1 \cdot 10^{-3}$	12
Sodium acetate	$5 \cdot 10^{-3}$	110
Choline chloride	$1 \cdot 10^{-4}$	92
	$5 \cdot 10^{-4}$	76
	$1 \cdot 10^{-3}$	65
KCl	$1 \cdot 10^{-3}$	105
$MgCl_2$	$1 \cdot 10^{-3}$	108
$CaCl_2$	$1 \cdot 10^{-3}$	103
$MnCl_2$	$1 \cdot 10^{-3}$	107
EDTA	$1 \cdot 10^{-3}$	104
Eserine*	$1 \cdot 10^{-5}$	0
Decamethonium	$1 \cdot 10^{-3}$	0
Trimethylphenylammonium	$1 \cdot 10^{-3}$	9
(+)-Tubocurarine	$1 \cdot 10^{-3}$	4
Gallamine triethiodide	$1 \cdot 10^{-3}$	10
Procaine	$1 \cdot 10^{-3}$	29
Dibucaine	$1 \cdot 10^{-3}$	43
Quinidine sulfate	$1 \cdot 10^{-3}$	62
Atropine sulfate	$1 \cdot 10^{-3}$	75

* Inhibition was found to be irreversible.

platelets. This is expected since platelets prepared by gel filtration should have less plasma protein contamination than platelets prepared by simple washing procedure. It can also be seen in Table II that acetylthiocholine and buturylthiocholine which are utilized as substrates in the colorimetric assays of cholinesterase[5,11] inhibit acetylcholine hydrolysis indicating that the same catalytic site may be involved; whereas the coloring agent 5,5′-dithiobis-(2-nitrobenzoic acid) has had no effect. The strong inhibition by agents such as eserine, decamethonium, trimethylammonium, (+)-tubocurarine, and gallamine triethiodide may indicate the presence of a separate esterase site and receptor site for the platelet enzyme[12]. Moderate inhibition resulted from use of other anticholinesterase agents such as procaine, dibucaine, quinidine sulfate, and atropine sulfate.

The colorimetric method of MacQueen *et al.*[11] was performed to compare the sensitivity of this method for measuring platelets cholinesterase activities and also as a check on the validity of the radiometric method. The final concentration of acetylthiocholine (or butyrylthiocholine) and 5,5′-dithiobis-(2-nitrobenzoic acid) in the reaction mixture were $1.0 \cdot 10^{-2}$ and $3.2 \cdot 10^{-3}$ M, respectively. The pH of the reaction mixture was 8.0 in 0.05 M Tris and the same amount of platelets ($2 \cdot 10^8$) was used for each determination. In this procedure, however, after the enzymatic reaction was retarded with quinidine sulfate, the platelets were removed by centrifugation (5000 × *g*, 5 min) and the absorbance of the supernatant fluid was measured in a Zeiss PMQ II spectrophotometer at 412 nm. The proper controls were determined

TABLE III

COLORIMETRIC DETERMINATION OF CHOLINESTERASE ACTIVITIES OF PLATELETS

Data were obtained by using acetylthiocholine (final concn $1 \cdot 10^{-2}$ M). Other assay conditions are as described in text. A molar extinction coefficient of $1.36 \cdot 10^4$ was used to calculate the activity (μmoles acetylthiocholine hydrolyzed per min at 37 °C, pH 8.0) (*cf.* ref. 5).
Calculation of enzyme activity:

Total ΔA for esterases activity = a−c−d = 0.160 or $4.7 \cdot 10^{-4}$ μmole/min.

ΔA for noncholinesterase activities (or esterase activity not inhibited by eserine) = b−c−d = 0.088 or $2.6 \cdot 10^{-4}$ μmole/min.

ΔA for cholinesterase activity = a−b = 0.072 or $2.1 \cdot 10^{-4}$ μmole/min.

Components in assay mixture	$A_{412\,nm}$ *(37 °C, 30 min)*	*% of total A*
(a) Platelets + acetylthiocholine + 5,5′-dithiobis-(2-nitrobenzoic acid)	0.822	100
(b) Platelets + acetylthiocholine + 5,5′-dithiobis-(2-nitrobenzoic acid) + eserine	0.750	92
(c) Acetylthiocholine + 5,5′-dithiobis-(2-nitrobenzoic acid)	0.320	39
(d) Platelets + 5,5′-dithiobis-(2-nitrobenzoic acid) (± eserine)*	0.342	41

* Eserine was found to have no effect.

simultaneously. An example is illustrated in Table III. It can be seen that because of the intense color contributed by the reagent blank (c, 39%), the enzyme blank (d, 41%) and an esterase(s) activity not inhibited by eserine ((b−c−d), 10%) (*e.g.* arylesterase[4], tributyrinase[13] and possible other esterases[14]), only a small fraction ((a−b), 8%) was directly obtained from the cholinesterase activity of the human platelets. Nevertheless, a comparison of data obtained by each of these two assay methods (Table IV) indicates these data to be in fairly good agreement. Substrate inhibition by acetylthiocholine or butyrylthiocholine was not observed in the colorimetric procedure.

TABLE IV

COMPARISON OF RADIOMETRIC AND COLORIMETRIC DETERMINATION OF THE CHOLINESTERASE ACTIVITIES OF HUMAN PLATELETS AND ENZYMES FROM OTHER SOURCES

The radiometric method used [$^{14}C_1$]acetylcholine ($2.7 \cdot 10^{-4}$ M) as substrate. The colorimetric method used acetylthiocholine ($1.0 \cdot 10^{-2}$ M) as substrate. The same amount of platelets ($2 \cdot 10^8$) was used in both methods. Other conditions are as described in text. Human erythrocyte membrane was prepared as described by Dodge *et al.*[15]. The enzyme activities from other sources were analyzed under the same conditions, and ratios were obtained from their respective rates based on the same amount of enzyme used.

Enzyme sources	*Radiometric assay* ($\times 10^{-4}$ *μmole/min)*	*Colorimetric assay* ($\times 10^{-3}$ *μmole/min)*	*Colorimetric/ Radiometric*
Washed platelets	1.14	2.16	1.9
Gel platelets	1.28	2.09	1.6
Bovine erythrocyte acetylcholinesterase (Sigma)	1.91	2.02	1.1
Human erythrocyte membrane acetylcholinesterase	0.53	0.84	1.6
Horse serum butyrylcholinesterase (Sigma)	0.91	10.50	11.5
Human platelet-poor plasma butyrylcholinesterase	2.00	10.20	5.1

The results obtained by the radiometric method described above have demonstrated clearly that it is a most sensitive and specific method for the measurement of the cholinesterase activities of human platelets. It is obviously superior to the less sensitive and relatively nonspecific colorimetric method. Furthermore, kinetic data (Figs 1 and 2; Tables II and IV) and substrate specificity studies (Table I and ref. 16) suggest that the intact platelet cholinesterase is most likely an acetylcholinesterase.

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