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PURIFICATION AND CHARACTERIZATION OF BUTYRYLCHOLINE-HYDROLYZING ENZYME FROM *PSEUDOMONAS POLYCOLOR* *

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Summary

A butyrylcholine-hydrolyzing enzyme (EC 3.1.1.-) of *Pseudomonas polycolor* IFO 3918 was purified approximately 9270-fold with a recovery of 9.9% by use of chromatographic techniques. The enzyme preparation appeared to be homogeneous when subjected to electrophoretic and ultracentrifugational analyses. The molecular weight was determined as approximately 59 000 by gel filtration. Isoelectric focusing electrophoresis revealed that the enzyme had an isoelectric point around pH 5.1. The enzyme catalyzed the hydrolysis of butyrylcholine with the maximum activity among various esters tested, and split benzoylcholine, propionylcholine and some aliphatic esters, but did not attack acetylcholine. The estimated value of K_m at pH 7.5 and 25°C was $7 \cdot 10^{-4}$ M for butyrylcholine. The enzyme was irreversibly inhibited by organophosphorus compounds and carbamates, such as diisopropylphosphofluoridate and eserine. The enzyme was inhibited by some compounds, such as atropine and quinidine. Quaternary ammonium salts showed an inhibitory effect on the enzyme resembling co-operative inhibition.

Introduction

Previous reports [1—3] on microbial cholinesterase revealed that bacterial cholinesterase was present in some species belonging to the fluorescent group of pseudomonads, such as *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*.

The cholinesterase purified from extracts of induced cells of *P. aeruginosa* A-16 appeared to be homogeneous upon acrylamide gel electrophoresis and ultracentrifugation. The enzyme, which was considered to be acetylcholinester-

* For the preceding paper in the series, see refs. 1 and 4.

ase from its substrate specificity, hydrolyzed acetylthiocholine and acetylcholine at the highest rates among the various esters tested, but did not hydrolyse butyrylcholine [4].

The authors found the presence of butyrylcholine-hydrolyzing activity in extracts of *Pseudomonas polycolor*, which could grow on butyrylcholine as its carbon and nitrogen source [1].

With a view to extensive study of microbial cholinesterase, the purification and characterization of the butyrylcholine-hydrolyzing enzyme (EC 3.1.1.-) of *P. polycolor* were undertaken in this paper.

Materials and Methods

Materials. The following chemicals were purchased from the Boehringer Co., Mannheim: cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), catalase and alcohol dehydrogenase (yeast).

Microorganism and culture. P. polycolor IFO 3918 was used. Culture was carried out in medium containing 1.5% peptone, 0.3% yeast extract, 0.1% K_2HPO_4, 0.1% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% butyrylcholine chloride, at pH 7.0. After 24 h cultivation, cells were harvested by centrifugation, washed twice with physiological saline and stored at −20°C as frozen paste until processed.

Assay of enzyme activity. Enzyme activity was assayed by the titrimetric procedure, unless otherwise stated. Titrimetric measurement based on the acid formed by the hydrolysis of various esters was made using a Radiometer automatic titrator. The reaction mixture, adjusted to pH 7.5, containing 260 μmol of NaCl and a suitable amount of enzyme in a volume of 2.6 ml, was incubated at 25°C in a 3.0 ml reaction vessel in which gas phase had been replaced with nitrogen. The substrate (3—40 μmol) was added, then titration with 0.01—0.001 M NaOH was continued.

One unit of activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of butyric acid from 10 μmol of butyrylcholine in 1 min under the above conditions. Specific activity is defined as units/mg protein.

Protein determination. Protein was determined by the method of Lowry et al. [5], or from the absorbance at 280 nm.

Ultracentrifugal analysis. The purity and sedimentation coefficient were determined with a Spinco Model E ultacentrifuge at 20°C and 59 780 rev./min.

Molecular weight estimation. The molecular weight of the *Pseudomonas* enzyme was estimated by the method of Andrews [6], using a 2.5 × 150 cm column of Sephadex G-200 in potassium phosphate buffer (pH 7.4) containing 1 M NaCl. Cytochrome *c*, chymotrypsinogen A, egg albumin, alcohol dehydrogenase and catalase, all at 2 mg/ml were used as standards.

Disc gel electrophoresis. About 80 μg of enzyme were electrophoresed on polyacrylamide gels according to Davis [7]. Electrophoresis was carried out in a solution of Tris/glycine at pH 9.0 with a 7.5% total polyacrylamide gel. On completion of the electrophoresis run, the gel was stained with a solution of Amido-Black reagent.

Isoelectric focusing electrophoresis. Isoelectric focusing was performed using an LKB 110 ml column, which was set up according to the method of Vester-

berg and Svensson [8]. Experiments were run at 4°C for 45 h, using 0.8% Ampholite at pH in the range 3—10. The pH of each fraction was measured with an Hitachi-Horiba M-7 meter.

Results

Purification of the enzyme.

All steps were conducted at 5°C. Potassium phosphate buffer (pH 7.4) was used and centrifugation was carried out at 12 000 × *g* for 20 min, throughout the purification procedure.

1. Preparation of cell-free extract. The washed cells (about 650 g as dry weight), suspended in 3.0 l of 0.1 M buffer, were disrupted in 500 ml portions for 20 min with a Kaijo-denki 19 kHz ultrasonic oscillator. A cell-free extract was obtained by centrifugation.

2. Ammonium sulfate fractionation. The extract (2500 ml) was brought to 0.3% saturation by adding 570 g of solid $(NH_4)_2SO_4$ and centrifuged. To the supernatant solution was added solid $(NH_4)_2SO_4$ to bring it to 0.7% saturation, and the solution was centrifuged again. The resulting precipitate was suspended in 0.05 M buffer (2800 ml) and dialyzed against the same buffer for 48 h with 5 changes of the buffer.

3. Chromatography on CM-Sephadex. The dialysate was applied to a CM-Sephadex column (5.0 × 78 cm) equilibrated with 0.05 M buffer. After washing the column with 4 l of 0.1 M buffer, the enzyme was eluted with 0.1 M buffer containing 0.3 M NaCl. Active fractions (320 ml) were combined and then dialyzed against 0.1 M buffer.

4. Chromatography on hydroxyapatite. The dialyzed material was applied to a hydroxyapatite column (3 × 6 cm) equilibrated with 0.1 M buffer. After the column was washed with 150 ml of 0.1 M buffer, the enzyme was eluted with 0.1 M buffer containing 1 M NaCl. The active component was precipitated at 0.7 saturation of $(NH_4)_2SO_4$.

5. Chromatography on Sephadex G-150. The precipitate was dissolved in 1.5

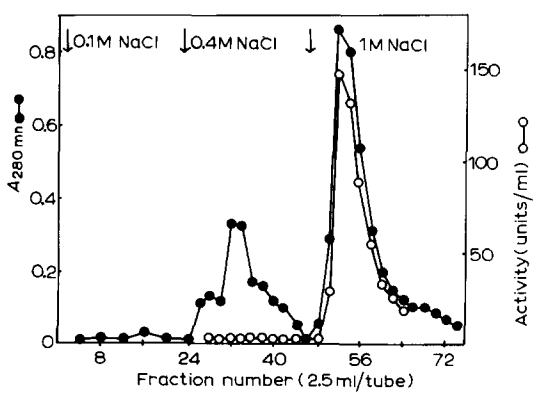

Fig. 1. Hydroxyapatite column chromatography pattern of the *Pseudomonas* enzyme. Preparation of butyrylcholine-hydrolyzing enzyme was rechromatographed on a column of hydroxyapatite as described in methods.

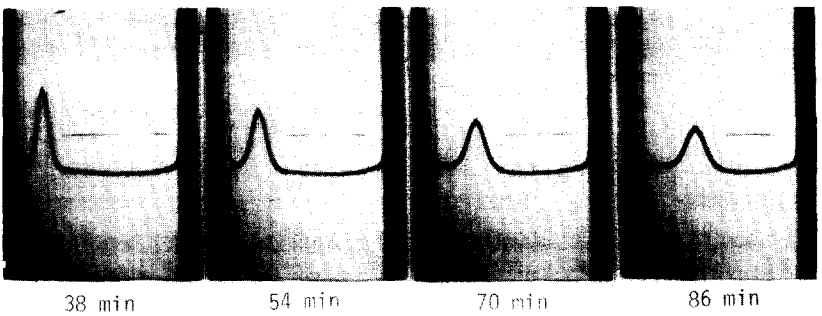

Fig. 2. Sedimentation patterns of the *Pseudomonas* enzyme with ultracentrifugation. Preparation had specific activity of 157.6 μmol/mg per min in phosphate buffer pH 7.4; the protein concentration was 6.4 mg/ml. Pictures were taken 38, 54, 70 and 86 min after top speed was reached (59 780 rev./min).

ml of 0.1 M buffer containing 1 M NaCl and the solution was placed on a Sephadex G-150 column (2.5 × 100 cm) which had been equilibrated with the buffer, and eluted at a flow rate of 6.5 ml/h. Active fractions were combined and concentrated by the addition of $(NH_4)_2SO_4$ to 0.7% saturation. The active component was again subjected to chromatography on the same Sephadex G-150 column. Fractions with a specific activity of 48 were pooled, and dialysed against 0.1 M buffer containing 0.1 M NaCl.

6. Chromatography on hydroxyapatite. The dialysate was applied to a hydroxyapatite column (1.6 × 5 cm) equilibrated with 0.1 M buffer containing 0.1 M NaCl. After washing the column with 50 ml of 0.1 M buffer containing 0.1—0.4 M NaCl, the enzyme was eluted with 0.1 M buffer containing 1.0 M NaCl (Fig. 1). Fractions (Nos. 50—60) with a specific activity of 155—160 were pooled and concentrated by the addition of $(NH_4)_2SO_4$ to 0.7% saturation.

The enzyme preparation (9 mg) obtained in an overall yield of 9.9% had 9270 times the specific activity of the original extract and was designated as the purified enzyme. A summary of the purification is shown in Table I.

TABLE I

PURIFICATION OF BUTYRYLCHOLINE-HYDROLYZING ENZYME FROM *PSEUDOMONAS POLYCOLOR*

Step	Volume (ml)	Total protein (mg)	Total activity * (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude extract	2500	866 400	14 728.8	0.017	1.0	100
Ammonium sulfate	280	271 600	11 678.8	0.043	2.5	81.6
CM-Sephadex	320	601	8 474.1	14.1	829.4	58.9
Hydroxyapatite	30	205	4 940.5	24.1	1417.6	34.3
Sephadex G-150 (I)	25	58	2 557.8	44.1	2594.1	17.9
Sephadex G-150 (II)	12.5	31	1 624.4	52.4	3082.3	11.3
Hydroxyapatite	7.5	9	1 418.4	157.6	9270.6	9.9

* Enzyme activity was assayed with butyrylcholine.

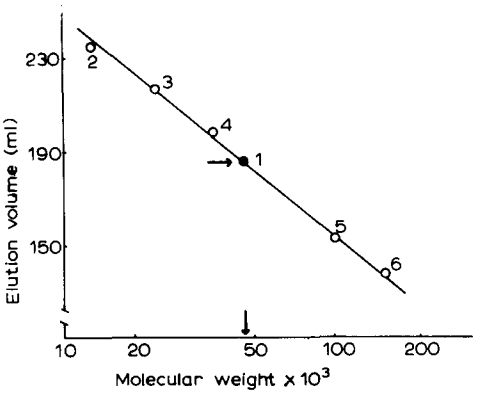

Fig. 3. Calibration curve of Sephadex G-200 column. The proteins used were (1) the *Pseudomonas* enzyme, (2) cytochrome *c* (M_r 13 000), (3) chymotrypsinogen A (M_r 25 000), (4) egg albumin (M_r 45 000), (5) yeast alcohol dehydrogenase (M_r 151 000), (6) catalase (M_r 240 000). Arrows: Elution volume and estimated molecular weight of butyrylcholine-hydrolyzing enzyme (average of three experiments).

Homogeneity.

The purified enzyme exhibited only one protein band on disc gel electrophoresis. Analysis of the purified protein in the analytical ultracentrifuge showed the presence of a single component (Fig. 2). Sedimentation coefficient $S_{20,w}$ was 2.5 S.

Molecular weight.

Molecular sieving on Sephadex G-200 was used to estimate the molecular weight of the purified enzyme. It is estimated that the molecular weight of the enzyme is about 59 000 (Fig. 3).

Isoelectric focusing electrophoresis.

The purified enzyme (2 mg) was subjected to ampholine electrophoresis to

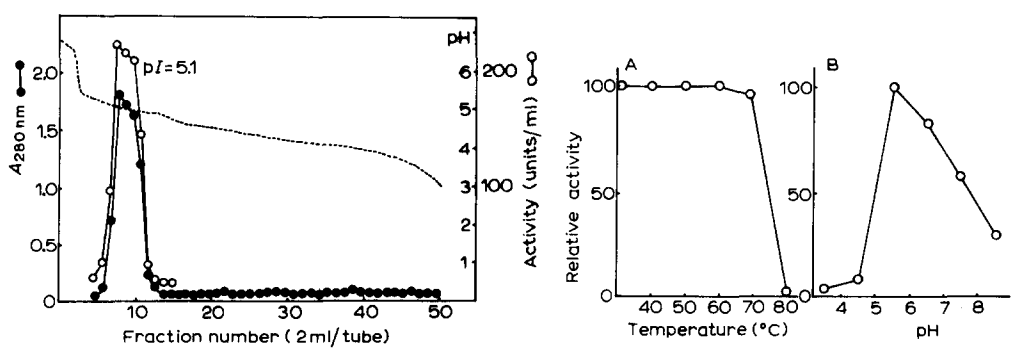

Fig. 4. Isoelectric focusing of butyrylcholine-hydrolyzing enzyme. Enzyme applied, 6.5 mg; 300 V, 3.5 mA for the first 10 h, then 700 V, 1 mA for the last 35 h at 4°C.

Fig. 5. Effects of temperature and pH on the stability of the *Pseudomonas* enzyme. (a) Termal stability curve. After incubation for 10 min at the temperature indicated, the remaining activity was assayed by the standard method. (b) After keeping at various pH values for 5 min at 75°C, each enzyme solution was adjusted to the optimum pH and residual activity was measured by the standard method.

determine the isoelectric point. The results indicate that the purified enzyme is homogeneous with an isoelectric point of pH 5.1 (Fig. 4).

Absorption spectrum.

The absorption spectrum of the enzyme in 0.1 M potassium phosphate buffer (pH 7.4) showed a maximum of absorbance only at 280 nm with a small shoulder at 290—292 nm.

Stability.

When the enzymes were dialyzed for several days against 0.1 M potassium phosphate buffer (pH 7.4) at 4°C, almost full activity was retained. When stored at −20°C in the same buffer solution, practically no loss of activity was observed during storage for at least 1 month.

Thermal stability of the enzyme was measured in 0.1 M phosphate buffer at pH 5.5 by heating at various temperatures for 10 min. As shown in Fig. 5A, the residual activity at 70°C was about 96%, and above 80°C the enzyme was completely inactivated.

When the enzyme solution was heated to 75°C for 5 min at various pH

TABLE II

RELATIVE RATES OF HYDROLYSIS OF VARIOUS CARBOXYLIC ESTERS BY PURIFIED BUTYRYLCHOLINE-HYDROLYZING ENZYME FROM *PSEUDOMONAS POLYCOLOR*

Reaction mixture (2.6 ml) contained 100 mM of NaCl, 5 mM of each substrate and the enzyme (0.12 units, specific activity 157.6 units/mg) in the presence of 1% acetonitrile. The activity, unless otherwise stated, was measured titrimetrically with 0.01 or 0.001 M NaOH at pH 7.5. The rate of hydrolysis for butyrylcholine was taken as 100.

Substrate	Relative activity (%) *
Butyrylcholine	100
Butyrylthiocholine	21.0
Propionylcholine	35.2
Propionylthiocholine	7.4
Benzoylcholine	23.0
Benzoylthiocholine	1.2
Methyl *n*-butyrate	1.1
Ethyl *n*-butyrate	9.5
n-Butyl *n*-butyrate	72.2
Isobutyl *n*-butyrate	30.5
n-Amyl *n*-butyrate	87.7
Isoamyl *n*-butyrate	50.8
Isoamyl propionate	17.2
Isobutyl propionate	6.1
Isoamyl isovalerate	6.3
Methyl *n*-valerate	1.6
Isobutyl isovalerate	3.7
n-Amyl acetate	1.7
n-Hexyl acetate	1.0
n-Butyl acetate	0.8

* Among the inactive substrates were: acetylcholine, acetylthiocholine, D,L-acetyl-β-methylcholine, succinylcholine, isoamyl acetate, ethyl *n*-caproate, methyl *n*-caprylate, ethyl pelargonate, methyl malonate, ethyl malonate, sec-butyl acetate, isobutyl acetate, ethyl propionate, isopropyl acetate, methyl acetate, ethyl acetate, ethyl formate, isobutyl formate and isoamyl formate.

values, the enzyme was stable between pH 5.5 and pH 7.0, but 90% and 60% of the original activity was lost at pH 4.5 and pH 8.5, respectively (Fig. 5B).

Effect of pH on rate of reaction.

The enzyme, examined in $Na_2CO_3/NaHCO_3$ (pH 9.5), glycine/NaOH (pH 9.0), K_2HPO_4/KH_2PO_4 (pH 5.5—8.0) and acetic acid/NaOH (pH 4.5—5.0) buffers, had an optimum reactivity in the pH range of 7.5—8.0 for the hydrolysis of butyrylcholine as substrate; below pH 5.5, the reactivity decreased markedly.

Substrate specificity.

The enzyme activity toward various substrates is presented in Table II. Maximum activity was obtained with butyrylcholine, butyrylthiocholine and propionylcholine among choline esters. Neither acetylcholine nor D,L-acetyl-β-methylcholine were hydrolyzed in the system used. The enzyme also catalyzed the hydrolysis of simple aliphatic esters containing carboxylic acids varying in chain length from 2 to 5 carbon atoms; butyryl esters were hydrolyzed at a greater rate than propionyl, valeryl or acetyl esters. With respect to alcohol moiety, a relatively high rate of hydrolysis with *n*-amyl, isoamyl and *n*-butyl esters was demonstrated. Accordingly, the presence of a polar alcohol moiety such as in choline esters might not be essential. Activity towards triolein as substrate was not detected, which suggested the absence of lipase activity.

The apparent Michaelis constant estimated from double reciprocal plots was $7 \cdot 10^{-4}$ M for butyrylcholine at pH 7.4 and 25°C. Butyrylcholine did not show marked substrate inhibition. Tests carried out in conjunction with studies on the effect of organophosphates on the *Pseudomonas* enzyme gave no evidence for phosphatase activity in the purified enzyme.

Inhibition by organophosphates and carbamates.

The *Pseudomonas* enzyme was inhibited by the organophosphorus compounds and carbamates tested (Table III). Inhibition progressed irreversibly, and was not reversed by the addition of acetylcholine to the reaction mixture. Activity of an enzyme preparation which had been completely inhibited by 10^{-3} M diisopropylphosphofluoridate or eserine was not recovered by overnight dialysis, a treatment which resulted in less than 7% loss in activity of an uninhibited preparation.

The reaction between these organophosphates and carbamates showed simple biomolecular kinetics, typical for other cholinesterases when the in-

TABLE III

INHIBITION OF THE *PSEUDOMONAS* ENZYME BY CARBAMATES AND ORGANOPHOSPHATES

Compound	Biomolecular rate constant ($mol^{-1} \cdot min^{-1}$)	Inhibition I_{50} (mM) *
Eserine	$4.1 \cdot 10^2$	$8.4 \cdot 10^{-2}$
Neostigmine	$1.5 \cdot 10$	2.4
Diisopropylphospho-fluoridate	$3.8 \cdot 10^3$	$9.2 \cdot 10^{-3}$
O,O'-Dimethyl-*O*,4-nitro-*m*-tolyl phosphate	$2.7 \cdot 10^3$	$1.3 \cdot 10^{-2}$

* I_{50} values: assay was done after incubation for 10 min at pH 7.5 and 25°C.

TABLE IV

INHIBITION EFFECT OF TROPINES AND MISCELLANEOUS COMPOUNDS ON THE *PSEUDOMONAS* ENZYME

Compound	K_i * (mM)	Inhibition type
Atropine	$2 \cdot 10^{-3}$	Competitive
Tropine	— **	—
Tropic acid	— **	—
L-Hyoscyamine	$7.2 \cdot 10^{-2}$	Competitive
Homatropine	$3.9 \cdot 10^{-2}$	Competitive
Scopolamine	$3.6 \cdot 10^{-1}$	Competative
Procaine	6.0	Competitive
Quinidine	$9.6 \cdot 10^{-1}$	Competitive
2-Pyridine aldoxime	— **	—
Curare	— **	—
Strychnine	— **	—
Nicotine	— **	—
Quinine	— **	—

* K_i values were calculated from Lineweaver-Burk plots.
** Inhibitory effect was barely observed at the concentration of 1 mM.

hibitor is in great excess. Biomolecular rate constants ranged from $3.8 \cdot 10^3$ $mol^{-1} \cdot min^{-1}$ for diisopropylphosphofluoridate and $2.7 \cdot 10^3$ $mol^{-1} \cdot min^{-1}$ for *O,O'*-dimethyl-*O*,4-nitro-*m*-tolyl phosphate to $4.1 \cdot 10^2$ $mol^{-1} \cdot min^{-1}$ for eserine. Neostigmine, a powerful inhibitor of animal cholinesterase, was found to be a comparatively weak inhibitor of the *Pseudomonas* enzyme.

TABLE V

INHIBITION OF THE *PSEUDOMONAS* ENZYME BY QUATERNARY AMMONIUM COMPOUNDS

Compound	I_{50} (mM) *
Tetramethylammonium iodide	12
Trimethylethylammonium iodide	2.5
Trimethylpropylammonium iodide	5.9
Trimethylbutylammonium iodide	9.2
Trimethylpentylammonium bromide	43
Trimethylhexylammonium iodide	2.0
Trimethylphenylammonium iodide	12
Trimethylphenethylammonium bromide	14
Triethylmethylammonium iodide	4.9
Tetraethylammonium iodide	2.4
Triethylpropylammonium iodide	26
Triethylbutylammonium iodide	10
Triethylpentylammonium bromide	17
Triethylhexylammonium iodide	1.2
Triethylphenylammonium iodide	0.5
Triethylbenzylammonium iodide	9.0
Tetrapropylammonium iodide	4.3
Tetrabutylammonium iodide	12
Dimethylphenylammonium iodide	1.3
Ethyltripropylammonium iodide	1

* The values for 50% inhibition by quaternary ammonium salts were determined under standard conditions, except that substrate concentration was 0.0015 M (butyrylcholine iodide).

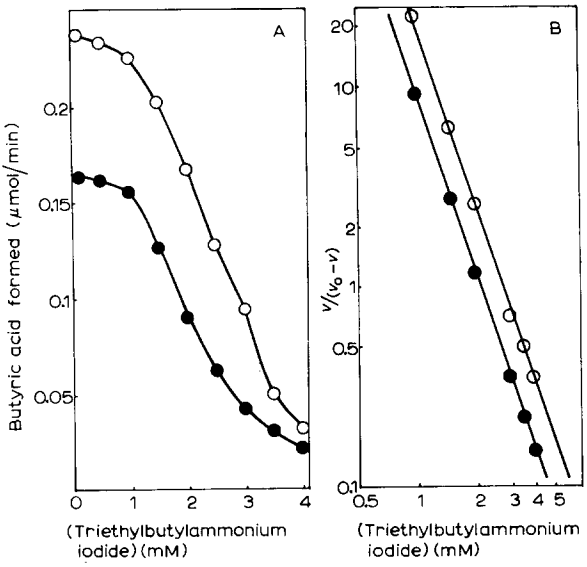

Fig. 6. The effect of changes in the concentration of triethylbutylammonium iodide on the rate of butyric acid by the *Pseudomonas* enzyme. Substrate concentration of $5 \cdot 10^{-4}$ M (○) and $2 \cdot 10^{-4}$ M (●) butyrylcholine.

Inhibition by tropine and miscellaneous compounds.

The *Pseudomonas* enzyme was competitively inhibited by atropine, homatropine and scopolamine (Table IV). The most potent inhibitor was atropine, which is the racemate of D,L-hyoscyamine, and the K_i value was $2.0 \cdot 10^{-6}$ M. Since the K_i value for L-hyoscyamine was 7.2×10^{-5} M, it appears likely that the enzyme has a stronger affinity for D-hyoscyamine than L-hyoscyamine. Tropine and tropic acid, components of atropine, did not show any measurable effect on its activity with butyrylcholine. Furthermore, the enzyme did not exhibit atropinase activity towards atropine. The enzyme was also inhibited by other pharmacologic compounds. Quinidine (K_i $9.6 \cdot 10^{-4}$ M) and procaine (K_i $6.0 \cdot 10^{-3}$ M) also caused competitive inhibition. The enzyme was not affected by quinine and F^- at concentrations of 1 mM, although these have long been known to inhibit unspecific esterase in the concentration range of 0.1—1 mM [9—11].

Inhibition by quaternary ammonium salts.

This type of inhibitor has been shown previously to be specific for two types of cholinesterase, true cholinesterase and pseudocholinesterase, but to have no effect on liver esterase [12]. The hydrolysis of butyrylcholine by the *Pseudomonas* enzyme was also inhibited by quaternary ammonium salts, and the effect was influenced by the size of the quaternary ammonium ion (Table V). The data of velocity against inhibition concentration was partially sigmoidal. A sigmoidal curve was observed with all quaternary ammonium ions. Fig. 6A shows the inhibitory effect of triethylbutylammonium ions. Fig. 6B shows the same data plotted as log $v/(v_0 - v)$ against log I according to the equation that Roy [13] proposed in the inhibition of arylamine sulfokinase by a representa-

tive 17-oxosteroid, 3β-methoxyandrost-5-en-17-one. The plot was linear, with a slope of 3.

Effect of metal ions and various reagents.

The effects of various metal ions and other compounds on the *Pseudomonas* enzyme were examined. After preincubating the enzyme with these reagents for 10 min at 25°C, the residual activity was assayed. Ca^{2+}, Co^{2+}, Mg^{2+}, Na^{+}, Al^{3+}, Fe^{3+}, Mn^{2+}, Li^{+} and Sn^{2+}, which were all employed as Cl^{-} salts, had little or no effect at the concentration of 1 mM. Neither chelating agents, such as EDTA, *o*-phenathroline, sodium diethyldithiocarbamate, cuprizone, 2,2'-bipyridyl and sodium azide, nor carbonyl reagents, such as phenylhydrazine and semicarbazine, caused inhibition of the enzyme activity at the concentration of 1 mM. However, $AgNO_3$, $CuSO_4$ and $HgCl_2$ caused 90—100% inhibition at the same concentration, and furthermore an appreciable loss in the enzyme activity was detected with SH-group reagents such as *p*-chloromercuribenzoate and iodoacetic acid.

Discussion

P. polycolor was capable of growing on butyrylcholine as sole carbon and nitrogen source [1]. The first step in the metabolism was assumed to be cleavage of the ester bond by esterase, and actually butyrylcholine-hydrolyzing activity was found in the cell extract of *P. polycolor*. First, the purification of butyrylcholine-hydrolyzing enzyme was extensively undertaken from the extract of *P. polycolor* grown on peptone medium containing 0.1% butyrylcholine chloride. Purification of the *Pseudomonas* enzyme to a homogeneous state involved laborious procedures and gave low yield. The enzyme split butyrylcholine with the maximum activity among various esters tested, and attacked benzoylcholine and propionylcholine. It seemed to resemble pseudocholinesterase, but unlike the pseudocholinesterase of mammalian tissues, did not hydrolyze acetylcholine [4], the active regions of these esterases must have different natures, but bear similar functional groups.

All esterases have been designated as A-, B- or C-esterases on the basis of their reaction with organophosphates [14—16]. The *Pseudomonas* enzyme was inhibited by organophosphates such as diisopropylphosphofluoridate and *O,O'*-dimethyl-*O*,4-nitro-*m*-tolyl phosphate, and consequently was considered to be a B-esterase. The sensitivity to inhibition by eserine may be used as a further method for distinguishing different types of B-esterase. All types of cholinesterase are completely inhibited by 10^{-5} M of eserine [10,11,18]. Low-eserine-sensitive esterases of this type attacking short-chain fatty acid esters are usually named aliesterases. The *Pseudomonas* enzyme was sensitive to eserine, but the inhibition occurred in relatively higher concentration compared with the activity of animal acetylcholinesterase or pseudocholinesterase.

The *Pseudomonas* enzyme was also sensitive to atropine, which is a cholinergic blocking agent and has been shown by both Brizin [19] and Vincent and Parant [20] to inhibit serum, erythrocyte and nerve (brain) cholinesterase. Furthermore, quaternary ammonium ions, which are specific inhibitors at the anionic site of acetylcholinesterase or pseudocholinesterase, and quinidine,

which is a specific inhibitor for the serum pseudocholinesterase [21], had an inhibitory effect on the *Pseudomonas* enzyme. Although the enzyme resembles pseudocholinesterase (EC 3.1.1.8) in these respects, it seems to be an aliesterase in that it exhibited a considerably broad substrate specificity, namely that it hydrolyzed some nonpolar aliphatic esters preferably, and showed low sensitivity to eserine. Therefore, it might be proper tentatively to designate the enzyme a butyrylcholine-hydrolyzing enzyme.

As the enzyme was not inhibited by chelating reagents or most metal ions, there was no question as to the importance of metal ions in the reaction systems of the enzyme. The enzyme was inhibited by organophosphates and carbamates, and consequently the active center of this enzyme was considered to contain serine residue. However, SH-group reagents such as heavy metals, *p*-chloromercuribenzoate and iodoacetic acid were also inhibitory to the enzyme. It is not clear at present whether a thiol group is present near the active center or is essential for enzyme activity, as has been shown for A-esterase, which is extremely sensitive to mercuric ion [22].

A partial inhibition of the *Pseudomonas* enzyme shown by cationic cholinesterase inhibitors seemed to support the presence of an anionic site in this enzyme. A sigmoidal curve in the plot of velocity against inhibitor concentration was observed. As pointed out by Roy [13] in the case of arylamine sulfokinase, such a curve seems to indicate a "co-operative inhibition effect"; several inhibitors interact with inhibitor-binding sites on the enzyme molecule. The plot of log $v/(v_0 - v)$ against log I was linear, with a slope of 3. However, the theoretical significance of such a plot is small, as it is based on the rather improbable assumption that one molecule of the enzyme can bind with n molecules of inhibitor to form a complex EI_n without the intermediary formation of EI, EI_2, etc. Accordingly, the sigmoidal nature of the curve is obscure at present.

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