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An Adaptive Bacterial Cholinesterase from a *Pseudomonas* Species

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SUMMARY: An organism belonging to the *Pseudomonas fluorescens* species-group has been isolated from fermenting cucumber by enrichment culture, using acetylcholine as the sole carbon source. This organism produces an adaptive cholinesterase.

Cells grown in tryptic digest broth have a very low cholinesterase activity. Growth for a few days in the presence of acetylcholine, choline, or Difco nutrient broth produces much higher cholinesterase levels. Cell-free preparations are also active.

The enzyme is not identical with either of the main types of animal cholinesterase. It is sensitive to prostigmine, is not inhibited by excess of substrate, and has a unique substrate specificity pattern.

The organism also produces two prostigmine-insensitive lipases, splitting triacetin and tributyrin, respectively.

Cholinesterases belonging to two major types have been found in a large number of animal species (Augustinsson, 1948). The nerve-type acetylcholinesterase is associated only with specific organ systems (nervous tissue, erythrocytes) which are limited to the animal kingdom. Its function in nervous tissue has been partially elucidated. The function of the plasma-type cholinesterase found in plasma and various organs remains obscure. Do similar enzymes exist in micro-organisms? If so, what is their function in the microbial cell, and are they identical with either of the main types of animal cholinesterase?

Previous attempts to find cholinesterases in bacteria have been made by testing certain pathogens for their ability to split acetylcholine. Esterases have been found in pneumococci (Bernouilli & Bloch, 1944), and one strain has been shown to hydrolyse acetylcholine, but the specificity of the enzyme has not been explored (Schaller, 1942). Several other micro-organisms have shown very low cholinesterase contents (Vincent & de Prat, 1945; de Prat, 1945).

The enrichment culture technique seems more promising because it allows one to select from a large number of bacterial species those capable of utilizing acetylcholine as a carbon source for growth. Some of these may be equipped with cholinesterase, while others may attack the acetylcholine molecule otherwise than by preliminary hydrolysis. Not all organisms possessing cholinesterase would be isolated, since those which require specific growth substances other than acetate and choline would not survive in the enrichment culture.

A suitable starting material for such an enrichment culture would be one

in which acetylcholine is present. Fermenting plant materials are known to contain acetylcholine, and Stephenson & Rowatt (1947) have reported the isolation from sauerkraut of lactobacilli capable of synthesizing acetylcholine. It seemed likely that such a natural environment might also harbour organisms able to utilize acetylcholine as a nutrient. In these experiments fermenting cucumber was used to start the enrichment culture.

METHODS

Isolation. Mashed cucumber was allowed to ferment in a closed bottle filled to the brim with tap water. Vigorous gas production was seen in a few days and the medium became acid. On the third day a transfer was made to a 25 ml. Erlenmeyer flask containing 0.1 % recrystallized acetylcholine (ACh) in 5 ml. of a mineral medium consisting of KH_2PO_4 0.1 %, NH_4Cl 0.1 %, and MgSO_4 0.05 %, at pH 6. After a second transfer in the same medium, the culture was plated out on Difco nutrient agar. All cultures were grown aerobically at room temperature. Solutions containing ACh were sterilized by filtration.

Growth and respiration. Growth experiments to determine which substrates could serve as carbon or nitrogen source were carried out in mineral medium with ACh bromide, sodium acetate, choline chloride, or glucose, added at 0.1 % concentration. When the substrate was to be tested as a nitrogen source, 0.1 % NaCl was substituted for the NH_4Cl in the mineral medium.

Respiration studies were done in the Warburg manometer at 27° with 20 % KOH in the centre well. The cells were harvested from nutrient agar plates and suspended in tap water. 0.2 ml. of the substrate was added from the sidearm after the rate of endogenous respiration had been established.

Esterase determinations were done manometrically at 27° in an atmosphere of 95 % N_2 + 5 % CO_2 . Intact cells or acetone-dried powders were suspended in bicarbonate Ringer solution (NaCl, 9 g.; KCl, 0.3 g.; CaCl_2 , 0.25 g.; NaHCO_3 , 2.1 g./l.; this solution is buffered to pH 7.3 when equilibrated with the gas mixture); 0.2 ml. of ACh or other ester was added from the sidearm. When prostigmine was used, it was added to the enzyme preparation in the vessel and incubated for at least 2½ hr. before addition of substrate.

Cell-free enzyme preparations. Cells were grown at room temperature in Roux bottles containing tryptic digest broth (beef heart digested with trypsin and filtered, with 0.1 % glucose added). Growth was heavy after 40 hr., and the culture had apparently reached the stationary phase of its growth. Acetone-dried powders were prepared by centrifuging the broth cultures and resuspending the cells twice in a large volume of acetone at 0°. The final centrifugate was allowed to dry at room temperature and ground in a mortar to a fine powder. The yield was 1.5–2 g./l. of broth. Weighed portions of powder (usually 50 mg.) were placed in Warburg vessels. With this weight of powder, duplicates gave good checks, but with larger amounts the suspensions were sometimes inhomogeneous. The powders have the disadvantage that there is considerable endogenous acid production before the ACh is added, but this could be decreased by incubation of the powder with the Ringer

solution for a few hours. Attempts to get the enzyme into solution from these preparations were abandoned because, on addition of water, a thick mucoid paste was formed, which could not easily be filtered or centrifuged.

RESULTS

Isolation and characterization. An organism was isolated on nutrient agar, which subsequently grew on ACh in mineral medium. It is a small, motile, Gram-negative rod, which grows on nutrient agar as a small, pale green, moist colony producing a green fluorescent pigment soluble in the medium.

Table 1. *Behaviour of the organism in routine tests*

Gelatin liquefaction	+	Gas production from sugars	—
Coagulation of milk	—	Indole production	—
Acid production from		NO ₃ reduction to NO ₂	+
Glucose	+	Growth on citrate	+
Lactose	—	Methyl red	—
Sucrose	—	Voges-Proskauer	—
Mannitol	—		
Salicin	—		

Pyocyanine was not detected when cultures grown on various media were extracted with chloroform in which pyocyanine is soluble but the fluorescent pigment is not. The organism grows well at room temperature but not at all at 37°. Further classification tests are summarized in Table 1. The organism is a member of the *Pseudomonas fluorescens* species group (Stanier, 1947). It has been deposited in the American Type Culture Collection (ATCC 11150) and the National Collection of Industrial Bacteria (NCIB 8286).

Growth and respiration. Media containing acetate as carbon source and ammonia as nitrogen source did not support growth. Choline could be utilized as both carbon and nitrogen source. Glucose is a satisfactory carbon source, though in unbuffered media the total growth is limited.

Fig. 1 shows that a suspension of the bacteria can oxidize ACh, as well as acetate and choline. ACh was utilized at the rate of about 0.6 μ mole ACh/hr. Since the spontaneous hydrolysis rate is less than 0.2 μ mole/hr., the organism could not have been merely oxidizing the products of spontaneous hydrolysis.

In the run shown in Fig. 1, 8 mole of oxygen were taken up per mole of ACh present, on the assumption that the total observed rate represents the sum of the endogenous rate and that due to ACh oxidation. In other experiments, values ranging from 6 to 8 mole of oxygen were obtained. Complete oxidation of ACh to CO₂ and NH₃ would use 9 mole of oxygen per mole of ACh.

Esterase

(1) *Demonstration of cholinesterase (ChE)*

Appreciable amounts of CO₂ are released from bicarbonate Ringer solution in the presence of ACh by suspensions of intact cells or cell-free preparations. Intact cells split about 2 μ mole of ACh/hr./50 mg. dry weight. Acetone-dried powders vary in activity from 1 to 5 μ mole of ACh split/hr./50 mg. The spontaneous hydrolysis rate of ACh was measured at concentrations of 0.02–2.0 %

and does not exceed $0.2 \mu\text{mole/hr.}$ Both with cells and with powders, some 'endogenous' CO_2 release, usually less than $1 \mu\text{mole/hr.}$, was seen before addition of the ester. Esterase activities, as given below, are corrected for this endogenous rate and for the spontaneous hydrolysis of the substrate.

Fig. 1. Respiration of bacterial suspension in tap water. The following amounts of substrates were added at 75 min.: ACh bromide, $3.4 \mu\text{mole}$; choline chloride, $3.4 \mu\text{mole}$; sodium acetate, $17 \mu\text{mole}$. A, 1 mole O_2 : 1 mole Ac; B, 5 mole O_2 : 1 mole Chol; C, 8 mole O_2 : 1 mole ACh.

Is the ChE essential for the utilization of ACh as a nutrient? The relative rates of oxidation and hydrolysis of the molecule supply a clue, for only if the ester can be hydrolysed faster than it can be oxidized may we postulate that hydrolysis is a necessary first step in its metabolism. Aliquot portions of a suspension of intact cells were tested with the following results:

Hydrolysis: $1.1 \mu\text{mole ACh hydrolysed/hr.}$

Oxidation: $6.3 \mu\text{mole O}_2$ taken up/hr. At 6–8 mole $\text{O}_2/\text{mole ACh}$ (see above) this represents $1.1\text{--}0.8 \mu\text{mole ACh oxidized/hr.}$

Thus it is reasonable to suppose that the organism attacks the ester by preliminary hydrolysis, followed by oxidation of the fragments.

(2) Adaptation

The esterase activity is determined in part by the medium in which the cells are grown. Initially, the organism was grown on nutrient agar, and active enzyme preparations were obtained. Later, tryptic digest media were used and no enzyme activity was found. However, the enzyme reappeared when the cells were grown in tryptic digest broth with added ACh (0.2%). Thereafter two separate cultures were maintained on tryptic digest agar, one with and one without added ACh.

The culture grown without ACh was used to test the time course of the adaptation (Table 2). After 48 hr. growth in broth without ACh an acetone-dried powder was prepared. It was inactive. This broth culture was used to inoculate flasks of ACh-containing broth from which powders were prepared at intervals during the next 3 days. In the first 24 hr. of growth there was hardly any enzyme activity, but in the course of the next 2 days the ChE appeared in amounts comparable to those of cultures grown for a long time on ACh media. Growth for 3 days in tryptic digest broth without ACh produced inactive preparations.

Table 2. *Adaptive cholinesterase activity of acetone-dried powders from cultures grown in various media*

ACh concentration in the esterase determinations: 0.2 %.

Medium	Run 1		Run 2		Run 3	
	Hours growth	Corr. rate*	Hours growth	Corr. rate	Hours growth	Corr. rate
Tryptic digest†	48	0.1	45	0.3	48	0.1
Tryptic digest + 0.2 % ACh	18	0.1	18	0.2	24	0.3
Tryptic digest + 0.2 % ACh	60	4.1	43	3.5	34	0.8
Tryptic digest + 0.2 % ACh	.	.	67	2.1	48	1.2
Tryptic digest + 0.2 % ACh	.	.	.	.	74	5.5
Tryptic digest + 0.2 % choline	.	.	67	1.8	48	0.5
Nutrient broth	.	.	67	2.2	48	0.4
Nutrient broth + 0.2 % ACh	.	.	67	9.7	48	6.2
Nutrient broth + 0.2 % ACh	.	.	.	.	74	11.0

* Expressed as $\mu\text{mole CO}_2/\text{hr.}/50 \text{ mg. powder}$.

† These cultures were used to inoculate the others in the same run.

Nutrient broth (with no additions) or tryptic digest broth containing 0.2 % choline were found to be almost as effective as ACh tryptic digest media in stimulating enzyme production. By far the most active preparations were obtained from nutrient broth with added ACh. Two cultures grown on ACh showed activities of 1.6 and 1.4 $\mu\text{mole CO}_2/\text{hr.}/50 \text{ mg. powder}$. A single 48 hr. subculture without ACh decreased the activities to 0.4 and 0.1, respectively. It would of course have been interesting to follow enzyme production on mineral medium with ACh, but this has not been done because of the poor growth on this medium.

(3) *Substrate specificity*

(a) Cultures grown with ACh. The substrate specificity pattern was studied for comparison with those of other cholinesterases. Table 3 shows the behaviour of adapted acetone-dried powders. The simple esters methyl and ethyl acetate or butyrate were not attacked by the enzyme. Of the four choline esters tested, butyryl- and benzoylcholine were not split. Acetyl- β -methylcholine was hydrolysed slightly more slowly than ACh, and propionylcholine (PrCh) nearly 4 times as fast as ACh. Nu 2017-1 [(3-acetoxyphenyl)-trimethyl ammonium iodide], an acetyl analogue of prostigmine, was split at about the

same rate as ACh. The structures of some of these compounds are shown in Fig. 2.

(b) Cultures grown without ACh. Unadapted acetone-dried powders showed no appreciable enzyme activity with ACh, acetyl- β -methylcholine, methyl

Table 3. *Substrate specificity*

Esterase activity of acetone-dried powders from cultures grown with ACh. Each powder was tested with ACh (0.2 % in the reaction mixture) and with one or more other substrates. The final column shows the relative rate of hydrolysis of each ester compared with ACh.

Substrate	Substrate % conc.	Spontaneous hydrolysis	Corrected rate $\mu\text{mole CO}_2/\text{hr.}$		Ratio: other substrate: ACh
		ACh	Other substrate		
Methyl acetate	0.1	0.1	2.8*	-0.2	Not split
Ethyl acetate	0.2	.	5.5	0.1†	Not split
Methyl butyrate	0.1	.	5.5	-0.3†	Not split
Ethyl butyrate	0.05	0.2	2.8*	-0.4	Not split
Acetyl- β -methylcholine Cl	0.2	0.4	3.3	2.0	0.6
PrCh Cl	0.2	0.1	3.9	14.6	3.7
PrCh Cl	0.2	0.1	1.8, 1.9	6.8, 7.3	3.8
PrCh Cl	0.2	0.1	7.0	27.8	4.0
Butyrylcholine Cl	0.2	0.1	3.9	-0.2	Not split
Butyrylcholine Cl	0.2	0.1	4.1	0.1	Not split
Benzoylcholine Cl	0.2	.	3.3	-0.4†	Not split
Nu 2017-1	0.2	0.2	3.3, 3.6	3.8, 3.8	1.1
Nu 2017-1	0.2	0.2	1.8, 1.9	2.9, 3.0	1.6
Triacetin	0.2	0.2	5.5	12.7	2.3
Triacetin	0.2	0.2	11.0	23.3	2.1
Tributyrin	(10.0)‡	0.2	11.0	11.6	1.1

* ACh 0.1 % in the reaction mixture.

† Not corrected for spontaneous hydrolysis.

‡ The sidearm contained 0.2 ml. of tributyrin, a liquid poorly miscible with water.

butyrate or ethyl acetate as substrates. In one 10 hr. run, intact cells showed a small but definite activity towards both ACh and PrCh. The corrected rates of CO_2 evolution were constant ($0.46 \mu\text{mole CO}_2/\text{hr.}$ for ACh and 2.28 for PrCh), giving a ratio of 1:5 which is approximately the same as that shown by adapted powders.

(4) *Effect of varying substrate concentration*

The rate of CO_2 production was essentially constant at different ACh concentrations in the range 4.5×10^{-3} to $8.9 \times 10^{-2} \text{M.}$ No 'inhibition by excess of substrate' was seen. These results are shown in Table 4, which also includes similar data for PrCh. With the latter substrate, enzyme activity decreased about 25 % with increasing substrate concentration from 5×10^{-3} to 10^{-1}M.

(5) *Inhibition by prostigmine*

Prostigmine was added to acetone-dried powder suspensions in Ringer solution and allowed to incubate for several hours before testing. As shown in Table 5, the esterase activity was completely abolished by prostigmine concentrations of 10^{-3} to 10^{-2} M., using either ACh or PrCh as substrate; partial inhibition (68 %) was produced by 10^{-6} M. in intact adapted cells.

Fig. 2. Structures of choline esters, prostigmine, and Nu 2017-1.

Table 4. *Effect of varying substrate concentration on cholinesterase activities of acetone-dried powders from cultures grown with ACh*

Powder no.	Substrate	Concentration (M.)	Corrected rate
			(μ mole CO_2 /hr./50 mg. powder)
1	ACh Br	4.5×10^{-3}	4.4
	ACh Br	8.9×10^{-3}	4.1
	ACh Br	4.5×10^{-2}	4.1
	ACh Br	8.9×10^{-2}	4.0
2	PrCh Cl	5.0×10^{-3}	22.1
	PrCh Cl	1.0×10^{-2}	19.7
	PrCh Cl	5.0×10^{-2}	17.2
	PrCh Cl	1.0×10^{-1}	16.1

(6) *Lipase activity*

Triacetin and tributyrin were used as substrates for various enzyme preparations with the following results.

(a) Adapted acetone-dried powders split both fats. Tributyrin is split at about the same rate as ACh (Tables 3 and 5), but apparently by another enzyme

since it is insensitive to prostigmine (Table 5). Triacetin is split about twice as fast as ACh and the whole triacetinase activity is prostigmine-sensitive. A further indication that the triacetin is split here only by the ChE is provided by a 'mixed-substrate experiment' using ACh and triacetin. Here the corrected rates were: ACh alone $5.5 \mu\text{mole CO}_2/\text{hr.}$, triacetin alone 12.7 , ACh + triacetin 5.9 . As the rates are not additive, it would appear that in an adapted powder ChE is the main enzyme splitting triacetin.

Table 5. *Inhibition by prostigmine of cholinesterase activity of acetone-dried powders from cultures grown with ACh*

Run no.	Substrate	Prostigmine concentration (M.)	Incubation time (hr.)	Corrected rate ($\mu\text{mole CO}_2/\text{hr.}$)	
				Controls	With prostigmine
1	ACh	1.5×10^{-3}	18	2.5, 2.6	-0.3, 0.7
2	ACh	5×10^{-3}	4	7.0	0
	PrCh	5×10^{-3}	4	27.8	0
3	ACh	8×10^{-3}	3.5	11.0	-0.6
	Triacetin	8×10^{-3}	3.5	23.3	0.3
	Tributyrin	8×10^{-3}	3.5	11.6	11.4

Substrate concentrations in reaction mixture: ACh, PrCh, and triacetin 0.2%; tributyrin 0.2 ml. in sidearm.

(b) An unadapted acetone-dried powder (without ChE) failed to split triacetin. Thus no acetone-dried powder shows any triacetinase activity other than that due to ChE.

(c) Unadapted intact cells, however, split triacetin as well as tributyrin, and this triacetinase activity was unaffected by prostigmine. Measurement of total CO_2 evolution for known amounts of triacetin showed that only two of the three ester links were hydrolysed. Cells grown in tryptic digest broth with 0.2% triacetin split triacetin (prostigmine-insensitive) but not ACh, i.e. triacetin, though a substrate of the adaptive ChE, failed to stimulate its production.

DISCUSSION

To further our understanding of the function of cholinesterases it would have been useful to discover a bacterial enzyme which was a necessary component of the cell metabolism under all conditions, possibly playing the same role in bacterial and animal cells. The bacterial enzyme here described, however, seems to be one which makes its contribution to the cell only when in a limited environment where an unusual nutrient must be made available. The enzyme is of interest because it is a new type of ChE and because the course of its adaptive formation is unusually slow.

Production of the enzyme

The enzyme is considered to be adaptive because the addition of ACh to the growth medium results in increased ChE activity of the culture. Certain other substances (choline and some factor in nutrient broth) also stimulate

enzyme production, but triacetin, a ChE substrate, does not. Since the increased activity of adapted cultures is seen in cell-free preparations, it cannot be simply explained on the basis of increased permeability to ACh.

The ChE appears in adapted cultures only at the end of a growth period of 2 or 3 days. Selection of ACh-utilizing mutants in the depleted medium might account for this, but such mutants should retain their high enzyme activity when subcultured into non-ACh media. We find, however, that a single subculture in tryptic digest broth suffices to abolish ChE activity almost completely. The delayed adaptation might be due to inhibition of enzyme production in the presence of substrates for the cell's constitutive enzymes, i.e. a 'diauxie' effect (Monod, 1947).

Properties of the enzyme

While the bacterial enzyme is in some respects similar to one or both major types of animal ChE, it is not identical with either (Table 6) (Augustinsson, 1948, 1949; Mendel & Hawkins, 1950).

Table 6. *Comparison of properties of animal and bacterial cholinesterases*

Enzyme Source	Nerve-type Nervous tissue Erythrocytes	Plasma-type Plasma Various organs	Bacterial <i>Ps. fluorescens</i>
Inhibition by prostigmine	+	+	+
Inhibition by excess of substrate	+	—	—
Rates of enzymic hydrolysis of choline esters:			
Fastest	Acetyl Propionyl Acetyl- β -methyl Butyryl	Butyryl Propionyl Acetyl Benzoyl	Propionyl Acetyl Acetyl- β -methyl (Butyryl)*
Slowest	(Benzoyl)*	(Acetyl- β -methyl)*	(Benzoyl)*

* Not split.

Both animal cholinesterases are inhibited by prostigmine at very low concentration, $<10^{-6}$ M. While concentration ranges were not explored here, the bacterial ChE was completely inhibited at 10^{-3} to 10^{-2} M., and partially inhibited at 10^{-6} M.

The effect of varying substrate concentration provides an important differentiating point between the two animal cholinesterases. The nerve enzyme shows the phenomenon of inhibition by excess of substrate, the activity rising to a maximum with increasing substrate and then decreasing at still higher concentration, to form a bell-shaped curve. The plasma enzyme shows a sigmoid curve, levelling off after a certain maximum enzyme activity is reached. The bacterial ChE does not show inhibition by excess of substrate over the range of concentrations where that phenomenon is seen with all known nerve type enzymes. Thus in this respect the bacterial enzyme resembles the plasma type.

Nerve and plasma cholinesterases have different substrate specificity patterns, and the bacterial enzyme is unlike either. The nerve type enzyme

splits ACh faster than other choline esters, and does not split benzoylcholine. The plasma enzyme attacks benzoylcholine but not acetyl- β -methylcholine, and splits both propionyl- and butyrylcholine faster than ACh. The bacterial enzyme clearly fits neither of these patterns. Like the nerve enzyme, it hydrolyses acetyl- β -methyl- but not benzoylcholine. It resembles the plasma enzyme in splitting PrCh faster than ACh, but differs in failing to split butyrylcholine. Thus substrates with more than two carbon atoms in the acyl group (butyryl- and benzoylcholine) are not attacked, while greater variation is tolerated in the alcohol portion (acetyl- β -methylcholine, Nu 2017-1, triacetin).

The bacteria appear to have three enzymes with lipase activity. Triacetin hydrolysis in powders is found only in association with the ChE (i.e. only in adapted powders). Its complete sensitivity to prostigmine, as well as the result of the mixed-substrate experiment show that the whole triacetinase activity of powders is due to ChE. Another enzyme must be present to account for the prostigmine-insensitive *triacetinase* activity of unadapted intact cells. This lipase is evidently destroyed by the acetone-drying procedure. Its effect on tributyrin is unknown. *Tributyrylase* activity is insensitive to prostigmine and is present in unadapted cells, hence cannot be ascribed to ChE. This lipase does not split triacetin, and its presence in powders further differentiates it from the triacetinase.

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