Studies on a Cholinesterase of *Pseudomonas fluorescens*. I. Enzyme Induction and the Metabolism of Acetylcholine*

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A study of the metabolism of acetylcholine in a strain of *Pseudomonas fluorescens* indicates that acetylcholine is metabolized via choline, betaine, dimethylglycine, sarcosine, and glycine. Glycine can be oxidized directly to CO_2 and water but cannot serve as the sole source of carbon. It might however serve as a one-carbon acceptor and thus be utilized for growth. Serine gives excellent growth as sole source of carbon. With the single exception of ethanolamine, one- and two-carbon compounds would not support growth as sole carbon source at the concentrations employed. At higher concentrations, acetate could also be made to support growth. With the exception of 2-pyridine aldoxime methiodide, all inducers of the cholinesterase were metabolites of acetylcholine and their effectiveness as inducers was inversely related to their metabolic distance from choline. The irreversible cholinesterase inhibitor neostigmine did not affect induction of cholinesterase by choline.

Goldstein and Goldstein (1953) isolated from an infusion mixture a *Pseudomonas fluorescens* (ATCC 11150) capable of growing upon acetylcholine as the sole source of carbon and/or nitrogen. The organism was able to grow equally well on choline and was subsequently shown to possess a choline-oxidizing system (Goldstein, 1959). The metabolism of acetylcholine by this organism has been further explored in this work, particularly in relation to the *N*-methyl groups which are absolutely vital to the ability of this bacterium to grow on acetylcholine and its metabolic products at the substrate concentrations used in this work.

D. B. Goldstein (1959) studied the relative effectiveness in inducing the synthesis of the cholinesterase[1] of several esters related to acetylcholine. That work suggested the choline portion of the molecule to be of central importance since even the product choline could induce the enzyme while acetyl-β-methylcholine, although an effective substrate, was not able to induce the enzyme. This work extends the survey by examining several metabolic products of acetylcholine for their relative effectiveness in inducing cholinesterase synthesis. The purification and properties of this cholinesterase are presented in Fitch (1963 a,b).

METHODS

The *Pseudomonas fluorescens* strain was maintained on nutrient agar slants.

The growth medium was 0.066 M sodium phosphate buffer at *p*H = 7.1 and contained 10^{-2} M KCl, 10^{-3} M Na_2SO_4, 4×10^{-4} M $MgSO_4$, and 0.05% NH_4Cl. This mineral medium was supplemented by an appropriate carbon source at 10^{-2} M unless otherwise indicated. The NH_4Cl was occasionally omitted to force the utilization of an alternative nitrogen source. The mineral medium was also employed in the Warburg studies of the ability of *P. fluorescens* to oxidize various one- and two-carbon compounds.

To ascertain whether a compound would support growth and/or induce cholinesterase, the culture was grown at room temperature in Roux bottles. In growth (or induction) rate experiments, the cultures were incubated at 30° on a rotary shaker in Erlenmeyer flasks fitted with side arms which fit a Klett colorimeter.

In contrast to an earlier report (Searle and Goldstein, 1962), the cells grew exponentially for more than 6 hours, increasing 10-fold in concentration during that period. An example is presented in Figure 1 which shows growth on choline. Generation time is the time required for the bacterial density to double. Growth was followed turbidimetrically according to the method of Searle and Goldstein (1962) on a Klett colorimeter using the No. 54 filter, chosen to reduce interference from the soluble yellow-green pigment excreted into the medium by cells. Provided that the Klett reading is ≤ 100, each Klett unit equals 2.1 μg dry wt of cells/ml of culture in log phase growth. At full growth there were approximately 3×10^7 viable cells/Klett unit and 1 mg protein/Klett unit.

Enzymatic activity was assayed in 0.1 M phosphate buffer at *p*H = 6.2. The substrate was the acetyl ester of 3-hydroxyphenyl trimethyl ammonium bromide (AcPN)[2] at 4×10^{-4} M. Figure 2 shows its structural relation to acetylcholine and neostigmine. The carbonyl group and the quaternary nitrogen are the same distance apart in each instance. Upon hydrolysis of the substrate, there is released acetic acid and the phenol portion of neostigmine (PN).

In Figure 3 are presented the spectra of AcPN and PN. AcPN has a λ_{max} = 256 and 265 mμ, and λ_{min} = 238 and 263 mμ. PN has acidic λ_{max} at 271 and λ_{min} at 239 mμ; basic λ_{max} at 238 and 292, and λ_{min} at 217 and 262 mμ; isobestic points at 223, 258, and 278 mμ. The difference spectrum at *p*H = 6.2 shows a λ_{max} at 273 mμ with an extinction coefficient of 2200. The optical density of the product PN at 273 mμ follows Beer's law and was used in assaying cholinesterase activity. PN has a $pK_a = 7.74$.[3]

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[1] The recommendation of the Report of the Commission on Enzymes (1961) for this enzyme's name and designation is 3.1.1.8 acylcholine acyl-hydrolase.

[2] The following abbreviations are used: PN, the phenolic moiety of neostigmine; AcPN, the acetyl ester of PN; DFP, diisopropylfluorophosphate; 2-PAM, 2-pyridine aldoxime methiodide.

[3] This value was determined spectrophotometrically and checked by titration in a Radiometer recording titrimeter at 25° and 10^{-3} M. The value by titration was $pK_a = 7.7$ with an accuracy of ± 0.1. This value may be compared to that of Wilson and Quan (1958) who report a value of 8.1. The discrepancy can probably be accounted for by a combination of the different conditions employed and the probable errors involved in the values.

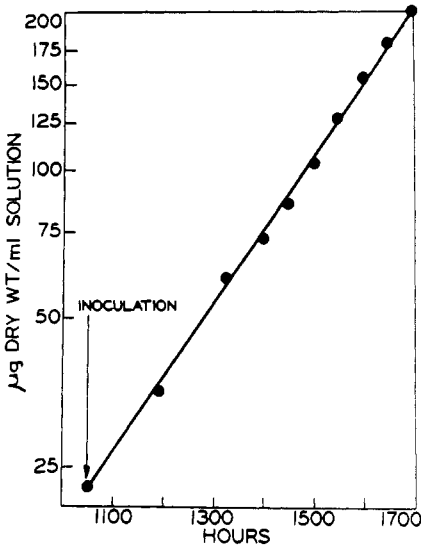

FIG. 1.—Rate of growth of *Pseudomonas fluorescens* on choline.

rate of AcPN hydrolysis will give a change in optical density of 3.4×10^{-4} units/minute.

The concentration of AcPN chosen provides nearly optimal velocity with very little change in rate from zero to 90% substrate utilization, i.e., zero-order kinetics are observed. However, this steadiness of reaction velocity is the result of the combined effect of the release of substrate inhibition as it is consumed simultaneously compensating for the increasing product inhibition as it accumulates.

RESULTS

Table I records the growth of this bacterium on twenty-two different substrates, each as sole source of carbon. The first three compounds are controls and include the lowest generation times found. Of the compounds related to the metabolism of acetylcholine, acetylcholine gives the greatest generation time of any compound studied. It can be seen that the further removed the substrate from acetylcholine, the more rapidly the bacterium grows until, with sarcosine, the generation time is nearly as low as with glucose or succinate. Neither acetate (as previously reported by

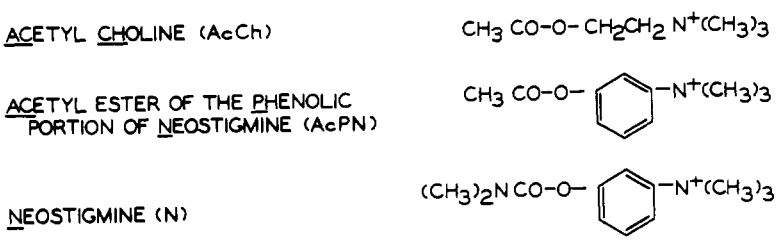

FIG. 2.—Comparison of structure of acetylcholine, the cholinesterase inhibitor neostigmine, and the artificial substrate acetyl ester of 3-hydroxyphenyl trimethyl ammonium ion.

Similar spectrophotometric methods for measuring cholinesterase activity have been presented before, using *p*-nitrophenyl acetate (Aldridge, 1953), benzoylcholine (Kalow *et al.*, 1956), β-carbonaphthoxycholine (Erdös and Debay, 1960), and *o*-nitrophenylbutyrate (Main *et al.*, 1961).

Activity was measured in whole cells since rupture of the cells by sonic oscillation did not appreciably affect cholinesterase activity. Goldstein (1959) previously observed a lack of effect of toluene on whole cell activity using acetylcholine as the substrate. For assay purposes, cells from full-grown cultures were centrifuged down and resuspended in 0.1 M phosphate buffer, *p*H = 6.2. The supernatant medium possessed no enzymatic activity. The reaction was started by the addition of AcPN and the absorbancy at 273 mμ was followed in a Beckman DK-2 recording spectrophotometer at 25°. Under these conditions, the nonenzymatic, spontaneous

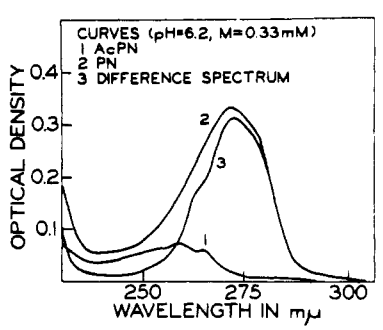

FIG. 3.—Spectra of substrate and product and their difference spectrum.

TABLE I
GROWTH-PROMOTING ACTIVITY OF VARIOUS COMPOUNDS[a]

	Compounds	Growth on Compound as: Sole Carbon Source	Sole Nitrogen Source	Oxidizable
1.	Glucose	1.5		
2.	Glycerol	2.2		
3.	Succinate	1.5		
4.	Acetate	—[b]		+
5.	Acetylcholine	2.8	+	
6.	Choline	2.0	+	
7.	Betaine	2.1		
8.	Dimethylglycine	+		
9.	Sarcosine	1.6	+	
10.	Glycine	−	+	+
11.	Dimethylamino-ethanol	−		
12.	Ethanolamine	+	+	
13.	Glyoxylate	−		+
14.	Formate	−		+
15.	Formaldehyde	−		+
16.	Methanol	−		−
17.	Ethanol	−		
18.	*n*-Propanol	+		
19.	*n*-Butanol	−		
20.	*n*-Amyl alcohol	+		
21.	*n*-Amyl acetate	−		
22.	Serine	1.5	+	

[a] All substrates at 10^{-2} M except methanol (0.25 M) and formaldehyde (0.12 M). Growth figures are generation times in hours, i.e., hours for bacterial density to double.
[b] Will support growth however at 0.04 M.

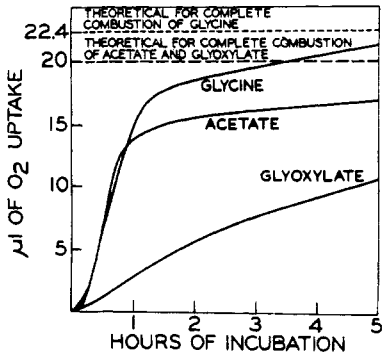

FIG. 4.—Time course of oxidation of compounds by *P. fluorescens*. Each Warburg vessel contained 1.0 ml 66 mM phosphate (*p*H = 7.1), 1.0 ml washed whole cells (~500 mg protein), 0.2 ml substrate, and 0.2 ml 3 N KOH in the center well. Sodium acetate = 50 mM, glycine = 75 mM, and glyoxylate = 100 mM. Endogenous respiration has been subtracted.

Searle and Goldstein, 1962) nor glycine would serve as sole carbon source for growth at the concentrations used here. No one or two carbon molecules (12–17) except ethanolamine were effective at these concentrations as the sole carbon source. Of a homologous series of alcohols (16–20), only propyl and amyl alcohols were effective carbon sources. Dimethylaminoethanol, though differing from choline by only one methyl group, was not utilized for growth as a carbon or nitrogen source. Both ethanolamine and serine supplied both carbon and nitrogen. Amyl acetate was not a carbon source although amyl alcohol was.

Den Dooren de Jong (1926), in extensive results on the ability of a number of organisms to grow on various compounds, found in accordance with the results reported here, that propyl and amyl alcohol, but neither butyl alcohol nor formate, support growth as sole carbon sources; that ethanolamine, betaine, and sarcosine could be used as the sole source of carbon and nitrogen but that glycine could serve only as a source of nitrogen. Of interest is the ability of *P. fluorescens* to grow on 0.1 M acetate, which we have confirmed. Growth occurs down to 0.04 M but not at the 0.01 M routinely used nor at 0.02 M.

There are three discrepancies where our work overlaps. Den Dooren de Jong obtained growth on methyl and ethyl alcohols (at 1%) and none whatsoever on choline either as a carbon source (0.036 M) or as a nitrogen source (0.005 M). The choline differences are undoubtedly the result of the selection of the particular strain isolated by the procedure of Goldstein and Goldstein (1953).

Several of the nitrogen-containing compounds can serve as the sole source of nitrogen. Thus glycine is further metabolized even if it is not suitable as a sole source of carbon. Indeed six one- and two-carbon compounds which do not alone support growth were tested to ascertain if they were metabolized, and all except methanol were oxidized. Figure 4 shows that whole cells (and it is also true of homogenates) metabolize acetate and glycine and do so more rapidly than they oxidize glyoxylate.

The effect of additional glucose and ammonia was studied to ascertain whether either carbon or nitrogen were limiting the growth rate. In a system containing only mineral salts (minus NH_4Cl) and substrate, the generation time was 2.8 and 2.0 hours with acetylcholine and choline, respectively, as substrate. The addition of either ammonia or glucose did not alter the generation time. There was no growth in the absence of substrate.

TABLE II
EFFECT OF VARIOUS COMPOUNDS AS INDUCERS OF CHOLINESTERASE

Compounds[a]	Induction[b]
Glucose	0.8
Glycerol	≤1
Succinate	≤1
Acetylcholine	85
Choline	100
Betaine	82
Sarcosine	10
Glycine	3,[c] 2[d]
Acetate	≤1
Amyl alcohol	≤1
2-Pyridine aldoxime methiodide	100
Ethanol amine	2
Dimethylamino-ethanol	≤1[e]

[a] All compounds at 10^{-2} M as sole carbon source unless otherwise indicated. [b] Induction relative to choline = 100 = 1.2 × 10^{-4} 273 mμ optical density units/min/Klett unit in full grown cultures. [c] NH_4Cl omitted, succinate present at 10^{-2} M. [d] NH_4Cl omitted, glycerol present at 10^{-2} M. [e] Succinate present at 10^{-2} M.

If both glucose and ammonia were added, the generation time fell to 1.5 hours and the presence or absence of substrate was immaterial. The failure of additional ammonia to increase growth rate on acetate plus choline was shown by Searle and Goldstein (1962).

For cells fully grown on the various substrates shown, Table II gives cholinesterase activity/Klett unit relative to the activity for cells grown on choline. All (except 2-PAM) of the compounds which had any inducing ability were members of the acetylcholine metabolic sequence. 2-PAM was the only compound as effective as choline in induction ability. All others were less effective inducers, their effectiveness diminishing the further the substrate was removed metabolically from choline.

The ability to induce cholinesterase is dependent upon other constituents of the medium (Table III). Both choline and acetylcholine induce when either ammonia or glucose is added but not when both are present. The activity observed in Table III, for cells growing exponentially, is the same as for fully grown cultures (see Table II) regardless of whether acetylcholine or choline is the inducer, if one compares the media containing NH_4^+. Induction was shown to be proportional to the increase in bacterial density.

TABLE III
ACTIVITY LEVELS OF CELLS INDUCED ON CHOLINE AND THE EFFECT OF ALTERNATIVELY AVAILABLE SOURCES OF CARBON AND NITROGEN[a]

Choline Medium plus Additions	Inocculum from Cells Grown in: Acetylcholine	Choline + Acetate	Choline
None	0.33	0.27	0.50[b]
+ Glucose	0.28	0.28	0.47
+ NH_4^+	0.40	0.47	0.50, 0.46
+ Glucose and NH_4^+	0	0	0

[a] Activity in change of 273 mμ OD units/5 min/10^3 Klett units of cells growing exponentially on the medium defined by the left-hand column. The inocculum was from a culture grown on mineral medium plus the carbon sources enumerated at the top of the remaining columns. The concentration of choline for the incubation in the first two columns = 0.025 M, and in the last column = 0.020 M. [b] Average of 5 determinations; standard error of mean ± 0.04.

TABLE IV
INDUCTION OF CHOLINESTERASE IN THE PRESENCE AND ABSENCE OF NEOSTIGMINE[a]

Growth Medium	Cholinesterase Activity	
	Untreated	2-PAM-treated
Succinate	0.0	0.0
Choline	0.56	[b]
Choline + Neostigmine	0.0	0.27

[a] Activity in change of 273 mμ OD units/5min/10^3 Klett units. Neostigmine = 0.2 M during growth. [b] It was shown using a purified cholinesterase preparation that the 2-PAM treatment did not affect the cholinesterase activity.

Choline induces even in the presence of the irreversible inhibitor neostigmine (Table IV). Although cells grown in the presence of neostigmine show no activity, treatment of those cells with 2-PAM reveals activity at least one-half as great as cells grown on choline alone. No such latent activity is revealed by 2-PAM treatment of succinate-grown cells.

DISCUSSION

Metabolism.—The choline oxidase system demonstrated by D. B. Goldstein (1959) could operate by first demethylating choline and then oxidizing the resultant ethanolamine or, alternatively, first oxidizing the choline and then demethylating the resultant betaine. The ability to grow upon all the intermediates involved in the second alternative clearly suggests this as the pathway by which choline is catabolized. Furthermore, the failure to grow upon dimethylaminoethanol indicates an inability (1) to oxidize the compound directly to dimethylglycine, (2) to continue the demethylation to ethanolamine, and (3) to remethylate it to choline, all three of the possible products being capable of supporting growth. A fourth possibility, the failure of dimethylaminoethanol to enter the cell, is very unlikely in view of the utilization of dimethylglycine as well as both choline and ethanolamine, the latter two compounds differing from dimethylaminoethanol by one more and two fewer *N*-methyl groups, respectively. It is therefore concluded that the metabolism of acetylcholine proceeds via choline, betaine, dimethylglycine, sarcosine, and glycine. The inability to grow upon the products acetate or glycine at these concentrations would suggest that the *N*-methyl groups are the essential carbon sources for growth on choline.

Is the growth rate on choline limited by the availability of the carbon or of the nitrogen? Since neither glucose nor ammonia individually affected the generation time, it must be concluded that the rate-limiting step controls a process necessary for the utilization of both the carbon and the nitrogen in choline and this step must therefore occur concomitantly with, or prior to, the utilization of either. The more rapid growth on sarcosine than on choline or betaine suggests that the rate-limiting step may be the utilization of the *N*-methyl groups.

The nature of the one-carbon acceptor is not known but it could be glycine. Thus glycine, though unable to serve as the sole source of carbon for growth, might be a partial source when acting as a one-carbon acceptor. Serine, the presumed product, provides the fastest growth rate of any member in the sequence. Rapid growth on sarcosine may be due to its being simultaneously both one-carbon donor and acceptor both in the proper one-to-one ratio. The poorer growth rate on betaine and choline then might be accounted for by the unfavorable three-to-one ratio of donors to acceptors. Additional glycine does not, by providing additional methyl acceptors, increase the growth rate on choline however since it apparently competes with the choline transport system drastically reducing the growth rate. Thus the generation time on 0.01 M choline + glycine is more than four times that on choline alone. A one-carbon donor, upon which the organism cannot grow, plus glycine, upon which the organism also cannot grow, but, in combination supporting growth, would show that glycine may act as a one-carbon acceptor. Among the nongrowth-supporting sources of one-carbon units are (Table I) dimethylaminoethanol, formaldehyde, formate, and bicarbonate. None has, in combination with glycine, so far proved effective in supporting growth.

Both acetate and glycine can be completely oxidized to CO_2 and H_2O. Glycine apparently is not transaminated (in contrast to *P. aeruginosa* as Bachrach, 1957, has shown), for both whole cells and homogenates metabolize glycine far more rapidly than glyoxylate (Fig. 4). Failure, at the concentrations tested, of glycine and acetate to support growth as sole carbon source suggests that they are oxidized without affording means of producing three- and four-carbon compounds. This is supported by the observation that alcohols with even numbers of carbon atoms do not alone support growth but propyl and amyl alcohols do. Acetate differs from glycine in that higher concentrations of acetate support growth but glycine will not serve as sole carbon source up to 0.1 M.

Ethanolamine was the only two-carbon compound which supported growth at the concentrations normally employed in this work, implying a special pathway for the utilization of this compound. Ethanolamine is normally formed by the decarboxylation of serine. The reversal of this pathway would permit growth but bicarbonate does not increase the growth rate on ethanolamine.

Induction.—The effectiveness of different compounds as inducers is presented in Table II, with the ability of choline to induce arbitrarily set at 100. No compound is more effective. The induction of the enzyme was directly proportional to the increase in bacterial density, acetylcholine, the substrate, being less effective than the product choline in inducing the enzyme, as shown by D. B. Goldstein (1959). The metabolic products of choline are also inducers, their effectiveness being reduced the further removed they are from choline. This is all consistent with the hypothesis of D. B. Goldstein (1959) that choline may be the specific inducer. It can not be decided whether the poor inducers are themselves less effective inducers or are less effectively converted to the inducer.

2-PAM is an effective inducer only because the bacterium can grow, albeit very slowly, on this compound. Table III shows that the enzyme is induced regardless of whether acetylcholine (or choline) is being used for either or both a carbon and a nitrogen source, but that when alternatives to both in the form of ammonia and glucose are available there is no induction. Neostigmine, an irreversible inhibitor of the *Pseudomonas* cholinesterase (Fitch, 1963a), does not support growth and as a consequence cannot be shown to induce the enzyme.

It has been suggested that active enzyme may be necessary to make additional enzyme (Spiegelman and Campbell, 1956). The experiment recorded in Table IV tends to deny this hypothesis, unless it be assumed that inhibited enzyme is equally acceptable for the induction process, for the results show that even in the presence of 0.2 M neostigmine, whose K_i for this enzyme is 10^{-6} M, 50% of normal activity was released upon

treatment with 2-PAM. This is not activity which would have been there in any event since 2-PAM fails to release any activity from cells grown on succinate. Furthermore, since the conditions utilized for release of activity do not give total release even in cell-free extracts, it must be concluded that neostigmine had no detectable inhibitory effect on the induction of cholinesterase.

A contrary conclusion has been reached by Durante (1958) using the ascidian *Ciona intestinalis*. Treatment of the unfertilized eggs with anticholinergics did not prevent normal muscular development following subsequent fertilization but the muscles were paralyzed and little cholinesterase activity could be detected, in marked contrast to untreated controls. She concludes that the inhibitors "block the original enzyme molecules in the unfertilized egg, thus rendering impossible their further production." However, cholinesterase activity was never demonstrable in the unfertilized egg and DFP, though bringing about the paralysis, failed to inhibit completely the formation of cholinesterase.

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ADDED IN PROOF

A repeat of the experiment in Figure 4 using glycine-1-C^{14} and glycine-2-C^{14} demonstrated that both carbon atoms are convertible to CO_2, thereby confirming the respiratory data which indicated that glycine may be metabolized completely to CO_2.

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Studies on a Cholinesterase of *Pseudomonas fluorescens*. II. Purification and Properties*

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An inducible cholinesterase from a strain of *Pseudomonas fluorescens* and purified 115-fold, was shown to have an energy of activation of 11,900 cal/mole below 31° and 7,400 cal/mole above. The enthalpy at 30° is 11,300 cal/mole. Using an artificial substrate, the acetyl ester of 3-hydroxyphenyl trimethylammonium bromide, a K_m of 5.6×10^{-6} M, was found. The substrate inhibits at high concentrations yielding an inhibition constant of 3.7×10^{-4} M. The product acetate does not inhibit at 1 M. The other product, 3-hydroxyphenyl trimethylammonium ion, shows mixed inhibition. Two of the inhibition constants for this product have been obtained. Neostigmine inhibits irreversibly but the enzyme may be reactivated with 2-pyridine aldoxime methiodide. Diisopropylfluorophosphate and tetraethylpyrophosphate do not inhibit this enzyme. Evidence is presented suggesting (1) that the enzyme has an ester-binding site containing special spatial features including an affinity for the *N*-methyl groups of choline, and a carboxyl group necessary in the ionized form for activity and (2) that an intermediate enzyme complex occurs in acetylated form.

A *Pseudomonas fluorescens* discovered by Goldstein and Goldstein (1953) provides a convenient source material for studies of an inducible cholinesterase[1] which has proved different in a number of respects from the nerve acetylcholinesterase of Wilson (1960) or from the blood cholinesterase studied by Augustinsson (1960). It is not, for example, inhibited by organic phosphates. Instead of a reversible inhibition by neostigmine, this enzyme is inhibited irreversibly. Furthermore 2-pyridine aldoxime methiodide (2-PAM)[2] reactivates the neostigmine-inhibited enzyme. This enzyme has been purified more than 115-fold and some of its properties

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