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.

ACKNOWLEDGMENT

The author is greatly indebted to Dr. Avram Goldstein for the sound suggestions, the constructive criticisms, and the gracious hospitality so thoughtfully rendered.

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*

WALTER M. FITCH†

From the Department of Pharmacology, Stanford University School of Medicine, Palo Alto, California

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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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† U. S. Public Health Service post-doctoral fellow CF-9087 (C2). Present address: Department of Physiological Chemistry, University of Wisconsin Medical School, Madison, Wisconsin.

[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 (1961).

[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.

TABLE I
PURIFICATION OF THE *Pseudomonas* CHOLINESTERASE

	Volume (ml)	Total Activity[a]	Protein (mg)	Specific Activity[b]	Purification	Per Cent Recovered
Original cell culture	11,000	495	537	0.92	1	100
Supernatant from 100,000 × *g* centrifugation for 1 hour of sonically ruptured cells	36	500	210	2.56	3	101
Ppt from 45–75% saturation with ammonium sulfate	10	220	9.0	24.5	27	44
Ppt from 45–75% saturation with acetone	5	169	1.6	105	115	34

[a] Total activity = (change in optical density at 273 mμ/10 min/ml of enzyme) per (volume of enzyme). [b] Specific activity = total activity/total mg protein.

are explored in this paper. Derivations for the kinetic parameters involved in substrate and product inhibition are presented in an appendix.

METHODS

Purification of the Enzyme.—Two enzyme preparations were used in this study. The less-purified preparation of the two had a specific activity 115 times that of the original cell culture. Its purification is presented in Table I. Protein was measured by the method of Lowry *et al.* (1951).

Assay of the Enzyme. –The general method was presented in a previous paper (Fitch, 1963a) but for the purpose of this study it was necessary to avoid substrate and product inhibitions. Therefore the concentration of acetyl ester of the phenolic moiety of neostigmine (AcPN) was reduced to 4×10^{-5} M, well below the inhibition constants of the substrate and product, and the lower optical density was compensated for by using 10-cm cuvets. The progress of the enzymatic reaction was followed continuously at 273 mμ in a Beckman DK-2 recording spectrophotometer. For any point on the curve there is an optical density related to the substrate concentration and a slope related to the velocity. Thus one progress curve affords many points for a Lineweaver-Burk plot (Lineweaver and Burk, 1934). There are no significant differences between velocities for substrate concentrations on the progress curve and initial velocities at the identical concentrations. Kalow *et al.* (1956) have previously reported the use of such continuous progress curves for determining kinetic values in their work on blood cholinesterases.

Where the effect of *p*H was determined, a single mixed buffer was used consisting of 0.029 M phosphate, 0.024 M citrate, and 0.025 M Tris. The cation was sodium. The velocities obtained in mixed buffer were comparable to those found in the usual phosphate buffer in the *p*H region in which both were tested, viz. 6.3–7.0.

RESULTS

Energy of Activation and Enthalpy.—In Figure 1 is an Arrhenius plot for this enzyme using AcPN as substrate. The energy of activation [$E = 2.303\,RT^2\,(d \log K/dT)$] is 11,900 cal/mole below 31° and 7,400 cal/mole above 31°. The heat of activation ($\Delta H^* = E - RT$) at 30° is then 11,900 − 600 = 11,300 cal/mole. This may be compared with the result of Wilson and Cabib (1956) who, using acetylcholine as a substrate and acetylcholinesterase from eels, found a ΔH^* = 14–19,000 cal/mole. A discontinuity occurs in the plot at a temperature of 31°. Except for the temperature at which they occur, similar discontinuities have been observed in lipase (Sizer and Josephson 1942), urease (Sizer 1939) (see, however, Kistiakowsky and Lumry, 1949), and fumarase (Massey 1953a,b).

Michaelis constant.—In Figure 2 may be seen a progress curve of an enzyme reaction with AcPN as the substrate at 2.8×10^{-5} M initial concentration. The cholinesterase concentration was sufficient to bring the hydrolysis to completion in less than 9 minutes. In Figure 3 is a Lineweaver-Burk plot showing a $K_m = 5.6 \times 10^{-6}$ M. Kalow and Davies report a K_m for human serum cholinesterase using benzoylcholine of 4×10^{-6} M (1959).

*Effect of p*H.—Figure 4 shows the effect of *p*H on the K_m of the enzyme. A similar plot of maximum velocity vs. *p*H is completely superimposable. The data, when fitted to a Dixon plot (1953), show a group with a pK_a at 4.6 important in the activity of the enzyme. The substrate has no pK in the range tested while the product has one at 7.7 (Fitch 1963a). Thus the pK represents a group in either the enzyme or its substrate com-

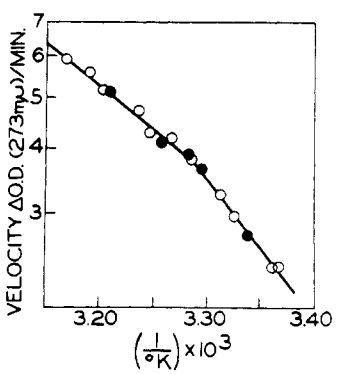

FIG. 1.—Arrhenius plot of *P. fluorescens* cholinesterase. Two experiments are shown. Buffer is 0.1 M phosphate (*p*H = 6.2); AcPN = 0.4 mM.

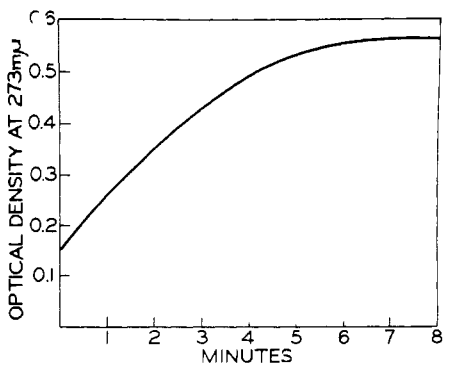

FIG. 2.—Progress curve for enzymatic release of 3-hydroxyphenyl trimethylammonium ion from its acetyl ester Conditions as for Fig. 1 except AcPN = 40 μM, temperature = 25°.

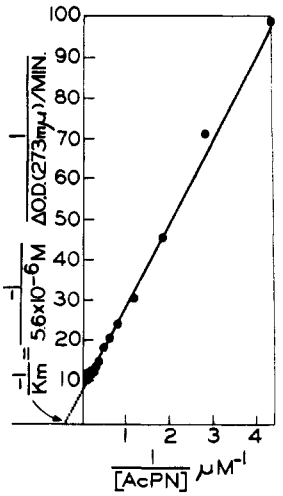

FIG. 3.—Lineweaver-Burk Plot of *P. fluorescens* cholinesterase. Conditions identical to those in Fig. 2.

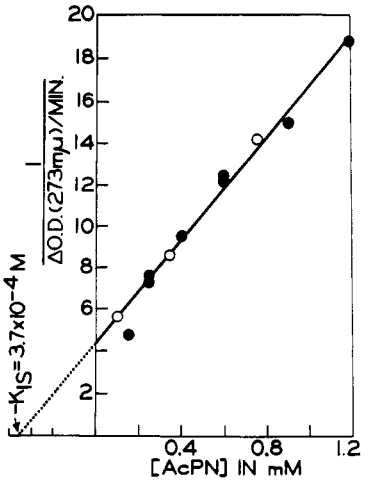

FIG. 5.—Substrate inhibition of *P. fluorescens* cholinesterase. Two experiments are shown. Buffer = 0.1 M phosphate at *p*H 6.2.

plex. The direction of the concavity in the Dixon plot indicates that the *pK* is for the enzyme-substrate complex. The *pK* value would indicate a possible distal carboxyl group of aspartate or glutamate. If so, the negatively charged carboxyl ion will exist almost exclusively above *p*H 5.6 (above which we have maximum velocity) and might well be the group attracting the quaternary nitrogen of the substrate. The data of Hase (1952) for horse serum cholinesterase yield an identical Dixon plot except that the *pK* is shifted to 7.7. Wilson and Bergmann (1950) found *pK* values at 7.2 and 9.3 for eel electroplax tissue.

Substrate Inhibition.—AcPN exhibits inhibitory properties at concentrations somewhat above the K_m and shows the general bell-shaped curve obtained when reaction velocity is plotted vs. log [AcPN]. Figure 5 shows a plot of $1/v$ vs. [AcPN] from which the inhibition constant for the substrate acting as an inhibitor (K_{is}) is determined to be 3.7×10^{-4} M. As explained in the appendix (equation 1), it cannot definitely be stated that this is the dissociation constant for the enzyme-bound double substrate. Indeed, Wilson and Alexander (1962) have recently adduced evidence indicating that the alternative (K_{as}) reaction occurs in nerve cholinesterase. Kalow *et al.* (1956) found an inhibition constant for benzoylcholine and serum cholinesterase equal to 10^{-4} M.

Product Inhibition.—Figure 6 shows several representative Lineweaver-Burk plots illustrating product inhibition of the enzyme. Such inhibition is reversible by dialysis. The uppermost curve, at the highest product

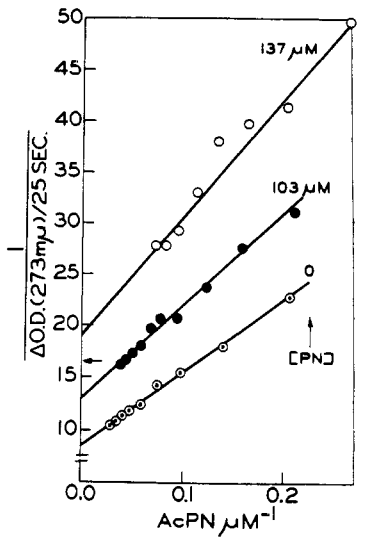

FIG. 6.—Product inhibition of *P. fluorescens* cholinesterase. Conditions as in Fig. 2.

concentration used (140 μM), shows the greatest scatter of points obtained in any experiment. The line is a least squares fit. Figure 7 shows a plot of the reciprocal of the apparent maximal velocity, $1/"V,"$ vs. product concentration (see discussion and equation 3 in the appendix). From this it is determined that $K_A = 1.9 \times 10^{-4}$ M.

It will be noticed that at the highest concentration studied, the reciprocal "*V*" deviates markedly (15%)

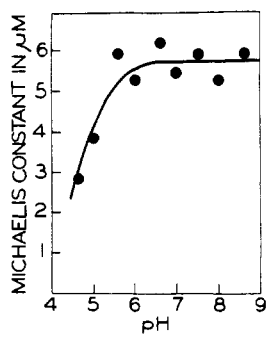

FIG. 4.—Effect of *p*H on Michaelis constant. Line was calculated as discussed in text. Buffer described in text, AcPN = 40 μM.

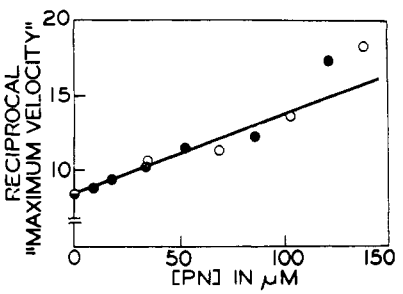

FIG. 7.—Effect of product concentration on the apparent maximal velocity. Two experiments are shown. Conditions as in Fig. 2.

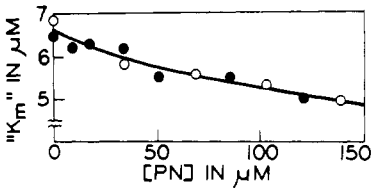

FIG. 8.—Effect of product concentration on the apparent Michaelis constant. Two experiments are shown. Line was calculated as discussed in text. Conditions as in Fig. 2.

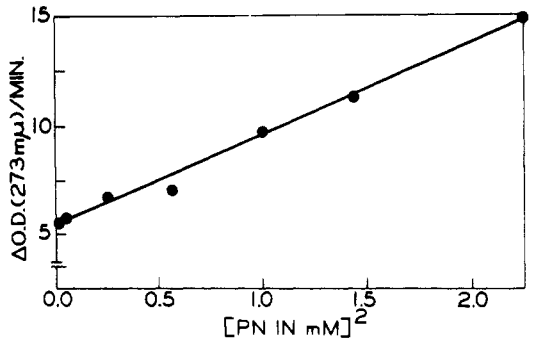

FIG. 9.—Effect of higher concentrations of products on cholinesterase activity. Conditions as in Fig. 2.

from the line drawn through the other points. The arrowhead on Figure 6 indicates where the intercept would have to be for the upper curve to produce a value on the curve in Figure 7. It would thus appear that product concentrations with powers greater than 1 may affect the maximum velocity.

In Fig. 8 is presented a plot of "K_m" vs. [PN] (see discussion and equation 4 in the appendix). K_C may be determined from any point on the graph and was calculated using $K_A = 1.9 \times 10^{-4}$ M, $K_m = 6.65 \times 10^{-6}$ M, and the experimental value of "K_m" (4.95×10^{-5} M) obtained at [PN] = 1.38×10^{-4} M. K_C was found to be 4.8×10^{-4} M. The smooth curve in Figure 8 is a plot of $6.65 - \text{"}K_m\text{"} = [PN]\,[(\text{"}K_m\text{"}/190) - (6.65/480)]$, equation (4) substituted with the known K values expressed in micromoles.

In Figure 9 is presented a plot of reciprocal v vs. $[PN]^2$ in accordance with equation (5) of the appendix. The value of the parenthetical term may be determined from the value of $[PN]^2$ where v is half maximal. In this case its value is 7.5×10^5 M^{-2}. A value for one of K_B or K_D would permit the calculation of the other.

The greatest concentration of PN shown in Figure 9 is 1.5×10^{-3} M. In the actual experiment, a value was also obtained for [PN] = 2×10^{-3} M. Its reciprocal velocity lay well above the curve drawn here (by a factor of nearly 2!) and would indicate that the velocity expression may be affected by product-concentration terms with powers greater than 2.

Inhibition affecting both the velocity and K_m terms as does the product inhibition here is frequently called *mixed* (Dixon and Webb, 1958) and has also been shown for nerve cholinesterase by Wilson and Alexander (1962) using various methylammonium ions.

The alternative product, acetate, does not inhibit this cholinesterase at concentrations up to 1 M.

Other Inhibitors.—In Table II are compounds tested for their ability to inhibit enzyme activity. The first group are known to react with specific amino acids in enzymes. Organic phosphates, possibly assisted by a histidine, are known to inhibit other cholinesterases by phosphorylating a serine group. Neither DFP nor tetraethylpyrophosphate however produced any inhibition although at much lower concentrations they subsequently produced 100% inhibition of serum cholinesterase. Barnard and Stein (1959) have shown bromoacetate to inactivate ribonuclease by reacting specifically with histidine. Bromoacetate had no effect on this cholinesterase.

TABLE II
INHIBITION OF CHOLINESTERASE BY VARIOUS COMPOUNDS[a]

Compound	Molarity	Per cent Inhibition
Organic phosphates and bromoacetate		
DFP (Diisopropylfluorophosphate)	5×10^{-3}	0
TePP (Tetraethylpyrophosphate)	10^{-3}	0
Bromoacetate	10^{-2}	0
Substrate-related compounds		
Choline	10^{-3}	50
Betaine	5×10^{-3}	50
Sarcosine	6×10^{-3}	0
Acetate	1	0
i-Amyl acetate	10^{-2}	0
Tetramethyl ammonium	4×10^{-3}	48
Tetraethyl ammonium	2×10^{-3}	86
2-Amino, 2-methyl propanol	3×10^{-2}	86
Cyclohexylamine	3×10^{-2}	86
Aniline	3×10^{-2}	91
Phenol 1 → 300 dil of H_2O satd soln		98
2-Pyrrolidone	4×10^{-2}	65
Carbamylcholine	10^{-5}	50
Pharmacologic agents and miscellaneous compounds		
Neostigmine	7×10^{-6}	89
d-Tubocurarine	5×10^{-4}	51
Physostigmine	10^{-3}	0
Ambenonium	4×10^{-4}	0
Atropine	3×10^{-2}	0
Amethopterin	3×10^{-5}	0
Tween 80, 1:300 (w/v)		0
Urea	3×10^{-2}	0
CN	7×10^{-3}	0
F	3×10^{-2}	60

[a] Concentration of AcPN = 4×10^{-4} M.

The second group shows the effect of various compounds related structurally or metabolically to the natural or artificial substrate of this enzyme. Only acetate, amyl acetate, and sarcosine fail to inhibit. By far the most potent inhibitor of the group is carbamylcholine. The inhibition, however, disappears overnight.

The third section shows the effect of other pharmacologic and miscellaneous compounds. Neostigmine is a very powerful inhibitor and its effect is not reversed by dialysis for 24 hours (also shown by Searle and Goldstein, 1962). The only other compound in the list showing inhibition is *d*-tubocurarine which Brzin (1955) has shown inhibits both serum and erythrocyte cholinesterase. Atropine, a cholinergic blocking agent, has been shown both by Brzin (1955) and by Vincent and Parant (1956) to inhibit serum, erythrocyte, and nerve (brain) cholinesterase at lower concentrations than those which proved ineffective here. Amethopterin, a drug more commonly known for its folic acid antagonism, has been shown by Jacobson (1961) to inhibit an acetyl transferase found in pigeon liver. The relevance here lies in the fact that our *Pseudomonas* cholinesterase is capable of acting as an acetyl transferase (Fitch,

TABLE III
THE EFFECT OF 2-PAM IN REVERSING NEOSTIGMINE INHIBITION

Presence of Neostigmine Throughout the 3-hr Incubation	Presence of 2-PAM for Last Hour of the 3-hr Incubation		
	−	+	
−	240	230	
+	76	170	[94 units of activity reactivated]

[164 units of activity inhibited]

1963b). Our cholinesterase is not however affected by amethopterin.

Wilson (1959) has designed a reactivator, 2-pyridine aldoxime methiodide (2-PAM), which reverses the effects of organic phosphate inhibition for cholinesterase. Table III shows the effect of 2-PAM in overcoming the neostigmine inhibition of this enzyme. The top row shows that 2-PAM does not affect the uninhibited enzyme while the bottom row shows a 57% recovery of the inhibited activity. No attempt was made to find the optimal conditions for recovery of activity.

DISCUSSION

The first experiments attempted to discover in what ways this cholinesterase was like other known cholinesterases. Cholinesterase was long ago shown to be inactivated by organic phosphates and Shaffer *et al.* (1954) showed the phosphate could subsequently be isolated attached to a serine. Janz *et al.* (1959) have been elucidating the amino acid sequence in the peptide which contains the serine-phosphate. Histidine is apparently intimately involved in this process as well. Wilson and Bergmann (1950) and Hase (1952) all implicate histidine on the basis of *p*H-activity plots, and histidine has also been shown to be involved in similar enzymes on the basis of photoinactivation and bromoacetylation. However, neither inhibition studies with organic phosphates and bromoacetate, nor the *p*H–activity plot indicates serine or histidine to be of any importance in this enzyme. A pK_a of 4.6 for a group in the enzyme-substrate complex and necessary for enzyme activity was found. The value is much more typical of a distal carboxyl group than of a histidine and is accordingly suspected of belonging to an aspartate or a glutamate. Maximal activity occurs above *p*H = 5.6 where a carboxyl group would be ionized, a fact consistent with this group attracting the positively charged nitrogen of the substrate.

The substrate inhibition shows that a second molecule of AcPN combines with an enzyme complex. It is usually assumed that it is the Michaelis complex with which the substrate *combines* (the K_{ss} reaction of the appendix). This would imply a second binding site for AcPN but, as the appendix discussion makes clear, substrate inhibition could also occur by combination with the acetylated enzyme (the K_{as} reaction), in which case the substrate is simply combining at the same site at a time after the PN has been removed but before the acetyl group has been hydrolyzed off. Evidence for the occurrence of the K_{as} reaction in nerve cholinesterase has recently been put forward by Wilson and Alexander (1962).

The product inhibition effect also shows that a second molecule of PN combines with the enzyme, i.e., one in addition to the reverse of the product dissociation which occurs as the enzyme proceeds in the normal course of hydrolyzing the substrate. When PN combines with free enzyme, this introduces a p^2 term in the numerator of "K_m." This would not require a second binding site. Should however either the suspected p^2 term of "V" or the p^3 term of "K_m" prove real, then either binding with the Michaelis complex (K_{sp} reaction) or the E-PN complex (K_{pp} reaction), respectively, occurs, and a second binding site must be accepted. The kinetic data are too weak on this point however to suggest anything more than the possibility of its occurrence. Indeed, Krupka and Laidler (1961a) have put forward evidence regarding an erythrocyte acetyl cholinesterase which they interpret as specifically excluding combination with the Michaelis complex. The conclusion depends however upon the term $k_2\beta >> k_3\alpha$, though they state that the conclusion depends only on $k_2 >> k_3$ (see their expression 3). In addition to this misstatement of fact, they take $k_2/k_3 = 6$ as sufficient basis for the validity of $k_2 >> k_3$. Furthermore the value of 6 is not for their bovine erythrocyte enzyme but for eel nerve cholinesterase (Wilson and Cabib 1956). A similar conclusion regarding failure of substrate to combine with the Michaelis complex is drawn in the succeeding article by Krupka and Laidler (1961b).

The work of Hestrin (1950) suggests the existence of an acyl-enzyme intermediate on the basis of the rate of hydroxamate formation with various substrates. Inhibition of cholinesterase by carbamylation was suggested by Meyers and Kemp in 1954. Wilson *et al.* (1960, 1961) studied intensively the effects of various carbamyl derivatives and proved both that cholinesterase is acetylated and that inhibition by some drugs, such as neostigmine, is by carbamylation of the enzyme. Equation (2), which the data fit, is derived from a mechanism which is in accord with these facts. This mechanism is supported by the lack of inhibition by acetate since an acetylated cholinesterase is more likely to dissociate, for all practical purposes, irreversibly. A product which dissociates irreversibly must fail to inhibit and vice versa. This amounts to an irreversible transfer of the acetyl group to water permitting the omission of the k_{-3} term.

Betaine, a product of choline metabolism (Fitch, 1963a) is of interest as an inhibitor for it differs from choline only in the oxidation of an unchanged alcoholic group to a negatively charged carboxyl group. Its similarity to choline in inhibitory power would tend to indicate that the amino function is of greater importance than the alcoholic group for the binding of choline. Furthermore, the enzyme carboxyl group of the ester-binding site is not likely to be where the acetyl end of acetylcholine is normally situated, otherwise it would repel the betaine carboxyl, assuming betaine orients on cholinesterase in the same way as does choline.

Still further removed metabolically from the normal substrate is sarcosine which differs from betaine only in the removal of two of the three methyl groups from the amino nitrogen. This removal produces a molecule with very little inhibitory activity, again implicating the nature of the amino function as of major importance in the binding of substrate to the enzyme. Furthermore, since both sarcosine and the quaternary betaine carry a positive charge at this *p*H, an important feature for binding at the ester-binding site, in addition to the charge, must be the presence of the methyl groups on the amino nitrogen. A similar importance for *N*-methyl groups was found by Wilson for nerve cholinesterase (1952).

From this we conclude: (1) that the *Pseudomonas* cholinesterase has an ester-binding site and possibly

another site capable of binding, with inhibition, either the substrate or the product; (2) that the ester-binding site probably contains a distal carboxyl group from glutamate or aspartate which is necessary in the ionized form, possibly to attract the positively charged nitrogen of the substrate; (3) that the ester-binding site requires two or more methyl groups attached to the nitrogen for optimal binding; and (4) that one of the intermediate complexes is an acylated enzyme which breaks down irreversibly to liberate the acid.

APPENDIX

The following kinetic derivations are all of the Briggs-Haldane (1925) type, as we have no evidence that the K_m equals the dissociation constant for the substrate. There is one simplifying feature however. Since acetate cannot be shown to inhibit even at 1 M concentration, its displacement from the enzyme may be regarded as an irreversible step in the reaction sequence and therefore $k_{-3} = 0$.

The reactions then are:

1 $\quad \mathrm{E} + \mathrm{AcPN} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{EAcPN}$

2 $\quad \mathrm{EAcPN} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{PN} + \mathrm{EAc}$

3 $\quad \mathrm{EAc} \xrightarrow{k_3} \mathrm{E} + \mathrm{Ac}$

The velocity equation for [PN] = 0 is of the usual Michaelis-Menten form

$$v = \frac{V}{1 + \frac{K_m}{s}}$$

where $V = \frac{ek_2k_3}{k_2 + k_3}$ and $K_m = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)}$

Substrate inhibition is usually accounted for by the following reaction:

$$\mathrm{EAcPN} + \mathrm{AcPN} \underset{}{\overset{K_{ss}}{\rightleftarrows}} \mathrm{E(AcPN)_2}$$

Here K_{ss} is the dissociation constant of the second substrate molecule on the enzyme. (In general, the subscripts of equilibrium K values indicate the components attached to the enzyme.) If however a second molecule can attach when an entire substrate molecule is already on the enzyme, certainly it is possible that it may attach to any of the other intermediate complexes which have less than a whole substrate molecule attached. Hence, we have

$$\mathrm{EAc} + \mathrm{AcPN} \overset{K_{as}}{\rightleftarrows} \mathrm{EAc \cdot AcPN}$$

This leads to the following substrate inhibition, velocity equation:

$$v = \frac{V}{1 + \frac{K_m}{s} + \frac{s}{K_{is}}} \qquad (1)$$

where $K_{is} = \frac{1}{k_2 + k_3}\left(\frac{k_2}{K_{as}} + \frac{k_3}{K_{ss}}\right)$

This is kinetically indistinguishable from the usual substrate inhibition equation, and therefore, just as a K_m is not necessarily equal to the dissociation constant (K_s) of the first molecule of substrate, neither is a substrate inhibition constant (K_{is}) necessarily equal to the dissociation constant, K_{ss}, of the second molecule of substrate.

Product Inhibition.—We assume that the product may complex in a manner analogous to the substrate in every way, i.e., with the free enzyme and all intermediate complexes. The following additional reactions are therefore required:

$$\mathrm{E} + \mathrm{PN} \overset{K_p}{\rightleftarrows} \mathrm{EPN}$$

$$\mathrm{EAcPN} + \mathrm{PN} \overset{K_{sp}}{\rightleftarrows} \mathrm{EAcPN \cdot PN}$$

$$\mathrm{EPN} + \mathrm{PN} \overset{K_{pp}}{\rightleftarrows} \mathrm{E(PN)_2}$$

The combination of PN with EAc is of course already accounted for as the reverse of reaction 2. This leads to the following product-inhibition velocity equation:

$$v = \frac{V}{1 + \frac{p(K_t + K_{sp})}{K_vK_{sp}}\left[1 + \frac{P}{K_t + K_{sp}}\right]} \Bigg/ 1 + \frac{K_m}{s} \cdot \frac{1 + \frac{pK_s}{K_vK_{sp}}\left[1 + \frac{p}{K_p}\left(1 + \frac{p}{K_{pp}}\right)\right]}{1 + \frac{p(K_t + K_{sp})}{K_vK_{sp}}\left[1 + \frac{p}{K_t + K_{sp}}\right]} \qquad (2)$$

where the new terms are

$$K_t = \frac{k_3}{k_{-2}}; \quad K_v = \frac{k_2 + k_3}{k_{-2}}; \quad \text{and} \quad K_s = \frac{k_{-1}}{k_1}$$

Cleland (1963) has shown that the nature of product inhibition is important for distinguishing alternative mechanisms. The major features of the product-inhibition velocity equation are that (A) product inhibition is mixed; (B) maximum velocity may be affected by product as a function of its second power at high concentrations; (C) the numerator of the K_m term may be affected by product as a function of its third power at high concentrations.

For any given product concentration, there is an observed apparent maximum velocity "V" which is the velocity when the substrate concentration is infinite and is defined from the product inhibition velocity equation as follows:

$$\text{"}V\text{"} = \frac{V}{1 + \frac{p}{K_A} + \frac{p^2}{K_B}} \qquad (3)$$

where $K_A = \frac{K_vK_{sp}}{K_t + K_{sp}}$ and $K_B = K_vK_{sp}$

Obviously, when $p = 0$, "V" $= V$. A plot of $1/$"V" vs. [P] should be linear (for concentrations of P not too great to make p^2 the controlling term) with $1/V$ the intercept and $1/VK_A$ the slope. The intercept may be divided by the slope to yield a value for K_A. In the absence of the K_{sp} reaction, $K_A = K_v$.

In a similar manner we may define from the product inhibition velocity equations an apparent Michaelis constant "K_m" as the substrate concentration giving half the maximal velocity for any fixed concentration of PN.

Hence $$\text{"}K_m\text{"} = K_m\left(\frac{1 + \frac{p}{K_C} + \frac{p^2}{K_D} + \frac{p^3}{K_E}}{1 + \frac{p}{K_A} + \frac{p^2}{K_B}}\right)$$

For values of p such that p^2 and p^3 are negligible, the equation may be rewritten as follows:

$$\text{"}K_m\text{"} = K_m + p\left(\frac{K_m}{K_C} - \frac{\text{"}K_m\text{"}}{K_A}\right) \qquad (4)$$

where $\frac{1}{K_C} = \frac{K_s}{K_mK_v} + \frac{1}{K_p}$

Thus a plot of "K_m" vs. p will not be linear but, since

K_A can be obtained from the "V" plots, K_C can be obtained from such a plot. Note that if K_m and K_v are known, the dissociation constant for the substrate K_s would be obtainable from the function K_s/K_mK_v. Previous use of product inhibition to determine K_s has however overlooked the potential effect of the K_p term.

Taking the reciprocal of the product-inhibition velocity equation, removing terms in p and p^3, and rearranging yields:

$$\frac{1}{v} = \frac{1}{V} + \frac{p^2}{V}\left(\frac{K_m}{sK_D} + \frac{1}{K_B}\right) \qquad (5)$$

A linear plot of $1/v$ vs. p^2 means that the p and p^3 terms are negligible in the region of p studied. The slope however is a complex function not easily evaluated.

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The Synthesis of α-Cephalins by a New Procedure*

Erich Baer, Yasuyuki Suzuki, and John Blackwell

From the Subdepartment of Synthetic Chemistry in Relation to Medical Research, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada

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A new procedure for the synthesis of the racemic and optically active forms of both saturated and unsaturated α-cephalins has been developed. It is considerably shorter than any of the known procedures for the synthesis of cephalins because it does not require as starting materials α,β-diglycerides or derivatives thereof. The L-, D-, and DL-α-cephalins are obtained by phosphorylating D-, L-, or DL-acetone glycerol with phosphorus oxychloride and quinoline, esterifying the resulting acetone α-glycerylphosphoric acid dichlorides with 2′-hydroxyethylphthalimide, removing the acetone group by mild acid hydrolysis, acylating the barium salt of L-, D-, or DL-α-glycerylphosphoryl-2′-hydroxyethylphthalimide with a fatty acid chloride, and removing the phthaloyl group of the diacyl α-glycerylphosphoryl-2′-hydroxyethylphthalimides by hydrazinolysis. The preparation of L-α-(distearoyl)cephalin and L-α-(dipalmitoyl)cephalin is described.

The synthesis of cephalins possessing the α-structure and L configuration of the natural substances was first accomplished by Baer and co-workers (Baer *et al.*, 1951, 1952; Baer, 1957). The cephalins prepared by these authors possessed two identical saturated fatty acid substituents. Eight years later, Baer and Buchnea (1959) reported the first synthesis of an L-α-cephalin containing two identical unsaturated fatty acids.

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More recently, Baer and Buchnea (1961) described the first synthesis of two L-α-cephalins containing both a saturated and an unsaturated fatty acid. These (Baer *et al.*, 1951, 1952; Baer, 1957; Baer and Buchnea, 1959, 1961) and other procedures (Baylis *et al.*, 1958; Bevan and Malkin, 1951; Grün and Limpächer, 1927; Hirt and Berchtold, 1957; Hunter *et al.*, 1948; Rose, 1947; Shvets *et al.*, 1961) for the synthesis of α-cephalins require as starting materials α,β-diglycerides or suitably substituted derivatives thereof. Each diglyceride or derivative has to be prepared individually When one considers that the preparation of α,β-