THE ACCELERATION OF THE ACETYLCHOLINESTERASE CATALYZED HYDROLYSIS OF ACETYL FLUORIDE

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The active center of acetylcholinesterase is believed to contain two subsites. One of these, called the esteratic site, is where the hydrolytic reaction takes place; the other, the anionic site, serves to bind the quaternary ammonium function of the substrate, acetylcholine. Quaternary alkyl ammonium ions are known to function as reversible inhibitors of the hydrolysis of acetylcholine. This is consistent with this model of the active center, since it is required that both subsites be unoccupied in order that acetylcholine bind to and react with the enzyme.

Certain molecules, not possessing a quaternary ammonium function react with the esteratic site alone, thereby leaving the cationic site unoccupied. Typical of these compounds are methanesulfonyl fluoride and dimethylcarbamyl fluoride. They both react with the esteratic site to form enzymically inactive acyl-enzymes. Their acylation of the enzyme is not hindered in the presence of certain alkyl ammonium ions. On the contrary, their rates of acylation can be accelerated by as much as thirty times in the presence of

these ions (Kitz, R. and Wilson, I. B., 1963; Metzger, H. P. and Wilson, I. B., 1963). The following mechanism has been suggested for this phenomenon: The acid fluoride can bind and react at the esteratic site while, at the same time, the anionic site is occupied by the quaternary alkyl ammonium ion. The binding of the quaternary ammonium ion induces a change at the esteratic site which causes this site to react more rapidly with the acid fluoride. This kind of explanation is related to the ideas of "induced fit" (Koshland, 1959), but the extent of the change need not be so extensive as suggested in that theory. It is this sort of mechanism, then, which possibly causes quaternary ester substrates to have higher maximum velocities than non-quaternary alkyl ester substrates. It appears then, that to expect the acylation of acetylcholinesterase to be accelerated by an alkyl ammonium ion, the acylating agent in question must be small enough to permit the simultaneous binding of itself and the alkyl ammonium ion, at the binding site usually reserved for a single molecule of acetylcholine.

The methanesulfonyl enzyme does not react with water and therefore, methanesulfonyl fluoride must be considered as an inhibitor of this enzyme (Kitz, R. and Wilson, I. B., 1962). Since the dimethylcarbamyl-enzyme does hydrolyze, although very slowly, carbamates can be considered mechanistically identical with substrates (Wilson, I. B., et al, 1961). These compounds, however, have long been classed as inhibitors. The acceleration of the trypsin catalyzed hy-

drolysis of ethyl glycinate by amines (Inagami, 1964), would again be an illustration of the acceleration of the enzymic hydrolysis of an extremely poor substrate. A similar, though smaller effect, was observed when 9-aminoacridine was added to the chymotrypsin catalyzed hydrolysis of methyl phenaceturate (Wallace et al, 1966). In this paper we describe the enzyme catalyzed hydrolysis of the substrate, acetyl fluoride, and the acceleration of this reaction by two alkyl ammonium ions.

METHODS AND MATERIALS

Solutions of acetyl fluoride (purchased from Hynes Research, Durham, North Carolina) were made in acetone. Chromatographically purified eel acetylcholinesterase, having an activity of 1000 U/mg (1U = 1 μm of acetylcholine hydrolyzed per minute) was purchased from Worthington Biochemical Corporation of Freehold, New Jersey. Three ml of a solution of .01 M $CaCl_2$ and .05 M maleate buffer at pH 7.0 (measured at 25°C) was added to a jacketed flask and thermostatted at 11°C. Enzyme (from a stock solution at a concentration of 10 mg/ml in the same solvent) was added to the reaction medium. The reaction was started by adding a solution of acetyl fluoride in acetone so that the initial concentration of acetyl fluoride was .005 M. Acetyl fluoride was assayed for in the following way (Hestrin, S. J., 1949): At appropriate time intervals, one-half ml aliquots of the reaction medium were withdrawn by a Cornwall pipetting device

(Becton Dickinson Company, Rutherford, New Jersey) and immediately added to tubes containing 1 ml of 1 M hydroxylamine in 1.75 M NaOH. In this way any acetyl fluoride present is converted to acethydroxamic acid. After one minute, one-half ml of 4 M HCl is added, immediately followed by the addition of one-half ml of a soln of 0.37 M $FeCl_3$ in 0.1 M HCl. This converts the acethydroxamic acid into a colored Ferric complex which is assayed for colorimetrically at 535 mμ. In this way, a sample can be withdrawn from the reaction solution and added to strong base every thirty seconds.

RESULTS AND DISCUSSIONS

The first order rate constant for the aqueous hydrolysis of acetyl fluoride is 0.54 min.$^{-1}$. (See Figure 1) The rate constant is unchanged when the reaction is done in the presence of either .012 M tetramethylammonium bromide or .003 M tetraethylammonium bromide. The reaction rate also remains the same when chymotrypsin is added (0.25 mg/ml) or when both acetylcholinesterase (0.25 mg/ml) and its potent reversible competitive inhibitor, 3-hydroxyphenyl trimethylammonium bromide (3.5×10^{-5}M) are added together.

Very large concentrations of enzyme had to be used to demonstrate enzyme catalyzed hydrolysis because the spontaneous rate of hydrolysis is very high and also because acetyl fluoride is not as good a substrate as acetylcholine. In the presence of acetylcholinesterase (0.25 mg/ml), and without the reversible competitive inhibitor present, the

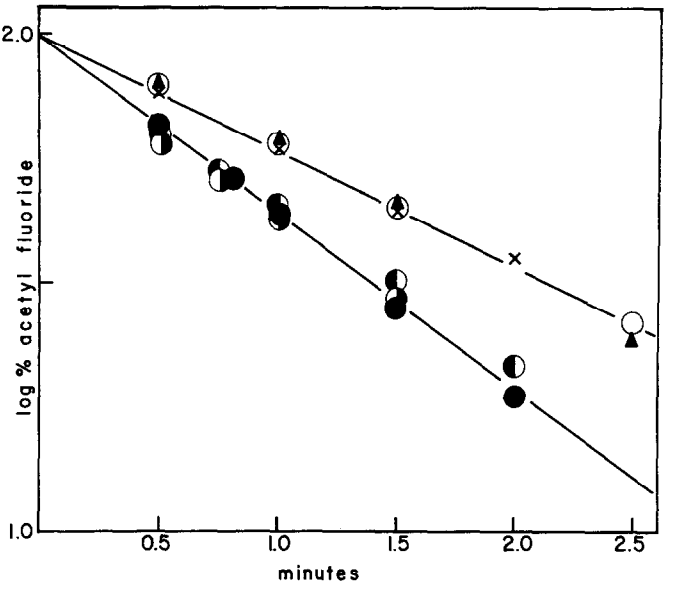

Figure 1: The aqueous and enzyme catalyzed hydrolysis of acetyl fluoride at 11°C, .01 M in $CaCl_2$ and .05 M in maleate buffer at pH 7.0. O = aqueous hydrolysis; ▲ = aqueous hydrolysis, .012 M in tetramethylammonium bromide; X = aqueous hydrolysis, .003 M in tetraethylammonium bromide; ● = enzyme catalyzed hydrolysis, 0.25 mg/ml acetylcholinesterase; ◑ = enzyme catalyzed hydrolysis, .08 mg/ml acetylcholinesterase and .012 M tetramethylammonium bromide; ◐ = enzyme catalyzed hydrolysis, .08 mg/ml acetylcholinesterase and .003 M tetraethylammonium bromide.

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measured first order constant for acetyl fluoride hydrolysis is increased 0.82 min.$^{-1}$. In the presence of one third the concentration of enzyme (.08 mg/ml) the rate of hydrolysis of acetyl fluoride is only slightly faster than the spontaneous rate, but in the presence of this amount of enzyme and either .012 M tetramethylammonium bromide or .003 M tetraethylammonium bromide the measured first order rate constant is increased to 0.82 min.$^{-1}$. (See Figure 1). Therefore, under these conditions, the quaternary ammonium ions can

provide a threefold acceleration of the enzymic hydrolysis of the substrate, acetyl fluoride.

In reactions where tetramethylammonium ion and tetraethylammonium ion function as accelerators, their binding constants (K_A) are substantially the same as their binding constants (K_I) when they function as reversible competitive inhibitors during acetylcholine hydrolysis (Kitz, R. and Wilson, I. B., 1963; and Metzger, H. P. and Wilson, I. B., 1963). The concentrations of each accelerator used in the work reported here are ten times larger than their K_A values. Therefore, the threefold increase of the enzymic reaction rate is probably close to the maximum value possible. Because of the very fast rates involved, it is not convenient to measure the maximum rate more exactly.

As suggested in the introduction, acetyl fluoride might be predicted to display the acceleration phenomenon if it should be a substrate for acetylcholinesterase. We have shown that it is and that its enzymic hydrolysis can be accelerated. This further supports the theory that binding at the anionic subsite causes a change within the enzyme so as to create a better catalytic center at the esteratic subsite.

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