SUMMARY

A kinetic analysis of the inhibition of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) by atropine is presented. It is shown that inhibition follows competitive kinetics at low concentrations of substrate (acetylthiocholine). At substrate concentrations above saturation atropine relieves the inhibition caused by excess substrate, but in this case, kinetic data are not of the competitive type. Rather, in the presence of atropine at high substrate concentration (acetylthiocholine iodide = 2.25 \times 10^{-3} \text{ M}), a plot of velocity versus substrate concentration changes into a sigmoid shape. The dependence of the reaction velocity on the atropine concentration is of an order less than 1 in the buffer studied and the substrate concentration below saturation (acetylthiocholine iodide = 7.5 \times 10^{-3} \text{ M}). At higher substrate concentrations (acetylthiocholine iodide = 7.5 \times 10^{-1} \text{ M}) the cooperativity of the inhibitor increases with n (the Hill coefficient) equal to 1.6.

Several reports (1-10) have suggested that cholinergic ligands bind to acetylcholinesterase at regulatory sites. For example Belleau and Di Tullio (1, 2) have shown that two distinct sites exist on the acetylcholinesterase molecule. One is the esteratic site which binds substrate; the second site binds \(d\)-tubocurarine and similar effectors. Changeux (3) showed that eel acetylcholinesterase strongly binds gallamine and \(d\)-tubocurarine at regulatory sites on the enzyme surface. This was confirmed by Kitz et al. (4). Changeux et al. (5) later demonstrated that acetylcholine binds to noncataytic sites as well as to the active center of the enzyme. We have shown that atropine and \((-\text{hyoscyamine})\) interact with squid enzyme at a region distinct from the active site (6, 7).

Atropine and a number of quaternary ammonium compounds accelerate the rate of hydrolysis of acetylcholine by acetylcholinesterase (10-15). The acceleration occurs at concentrations of substrate above saturation for the enzyme. Although very little evidence is available about the mechanism of this reaction, Roufogalis and Thomas (18) suggest that these compounds accelerate the deacetylation step in the hydrolysis.

In the present experiments we have investigated the effects of atropine, \((-\text{hyoscyamine}),\) and gallamine on the kinetic behavior of partially purified squid acetylcholinesterase, using acetylthiocholine as the substrate. The results suggest that atropine, \((-\text{hyoscyamine}),\) and gallamine protect the enzyme against substrate inhibition.

A preliminary report of some of these findings has appeared (10).

EXPERIMENTAL PROCEDURE

* Enzyme-Acetylcholinesterase was prepared from head ganglia of squid (Loligo opalescens) obtained from the Montreal Aquarium, Montreal, Canada. The enzyme was partially purified by the method of Kremzner and Wilson (16). The enzyme had a specific activity of 2.2 \(\mu\text{moles of acetylthiocholine hydrolyzed per min per mg of protein.}

* Assay Method The enzyme was assayed according to the method of Ellman et al. (17) with acetylthiocholine as substrate. The product of thiocholine reaction with 5,5'-dithiobis(2-nitrobenzoic acid) was determined on a Cary model 14 spectrophotometer. The absorbance at 412 nm was recorded as a function of time; a velocity of scanning of 2 inches per min was used. The rate of change of absorbance was linear over a period of 2 min for all concentrations of substrate and the slope was used to calculate the initial velocity.

* Enzyme activity was determined at 25° in a 1.0 ml reaction mixture containing 0.10 M phosphate buffer, pH 8.0, 0.01 M 5,5'-dithiobis(2-nitrobenzoic acid) containing sodium bicarbonate (1.5 mg per ml), acetylthiocholine, and inhibitors as indicated, with all reagents except acetylthiocholine (pH 7.0) adjusted to pH 8.0. All determinations were performed a minimum of four times.

* Chemicals-Chemicals were obtained from the following sources: acetylthiocholine iodide and 5,5'-dithiobis(2-nitrobenzoic acid), Sigma Chemical Company; atropine sulfate, atropine hydrochloride, and eserine (physostigmine) hydrochloride, Mann Research Laboratories; \((-\text{hyoscyamine})\) sulfate and eserine sulfate, Nutritional Biochemicals; neostigmine bromide, Pierce Chemical Company; gallamine triethiodide, gift from Poulenc Pharmaceutical Co.

RESULTS

Acetylcholinesterase when assayed at pH 8.0 exhibits Michaelis-Menten kinetics with respect to acetylthiocholine (Fig.
Fig. 1. Plot of initial reaction velocity (in micromoles of acetylthiocholine hydrolyzed per min per mg of protein) with respect to acetylthiocholine concentration in the presence of atropine sulfate. •—•, no addition; •—•, 1.0 mM atropine sulfate; △—△, 5.0 mM atropine sulfate; ■—■, 10.0 mM atropine sulfate. The reaction which was carried out in 1.0 ml was started by addition of acetylthiocholine to acetylcholinesterase. The reaction mixture also contained the following: 0.1 M phosphate buffer, pH 8.0, 0.01 M 5,5'-dithiobis(2-nitrobenzoic acid) containing bicarbonate, and atropine sulfate as indicated above. Other experimental conditions were given under "Experimental Procedure." Inset shows Lineweaver-Burk plots of the same data in the presence of two concentrations of atropine and by itself. The initial portions of the Michaelis curves ([S] = 15.0 to 225 μM) have been used for the Lineweaver-Burk plots. The lines were drawn by eye.

1. The $K_v$ value for acetylthiocholine as determined from Lineweaver-Burk plots (Fig. 1, inset) was $5.8 \times 10^{-5}$ M; the maximum velocity calculated as micromoles of acetylthiocholine hydrolyzed per mg per min was 2.15. At substrate concentrations above saturation, inhibition by excess substrate was apparent. The inhibitory portion of the curve is sigmoidal.

Fig. 1 also shows the saturation curve for acetylthiocholine in the presence of three concentrations of atropine sulfate. At low substrate concentrations and in the presence of inhibitor, the dependence of the velocity upon substrate concentration is hyperbolic and "appears" to level off and reach a saturating level. A further increase in the concentration of acetylthiocholine, however, produces a further increase in the velocity of the reaction. In the presence of high concentrations of substrate these plots assume a sigmoidal shape. Increasing the concentration of acetylthiocholine still further at a constant level of atropine relieves the inhibition by excess acetylthiocholine. Under present conditions the rate in the presence of atropine does not exceed $V_{max}$.

In Fig. 1 the data with atropine are also plotted according to the method of Lineweaver and Burk (18) and it may be seen that the kinetics at low substrate concentration obey the normal competitive form. The $K_v$ for atropine was $4.0 \times 10^{-3}$ M.

Other Inhibitors—Similar results were obtained with (−)-hyoscyamine sulfate and gallamine triethiodide. Saturation curves for acetylthiocholine at two different concentrations of (−)-hyoscyamine and gallamine triethiodide. Saturation curves for acetylthiocholine at two different concentrations of (−)-hyoscyamine and three different concentrations of gallamine are shown in Figs. 2 and 3, respectively. As with atropine, in the presence of (−)-hyoscyamine or gallamine, the dependence of velocity on substrate concentration is hyperbolic and seems to level off and reach a saturating level. A further increase in the concentration of acetylthiocholine, however, produces a further increase in the velocity. At very high substrate concentrations, both (−)-hyoscyamine and gallamine relieve the inhibition due to excess substrate. The inset figures show the Lineweaver-Burk plots. At low substrate concentrations, both inhibitors exhibit competitive kinetics. The calculated $K_v$ values for (−)-hyoscyamine and gallamine are $2.0 \times 10^{-2}$ M and $4.2 \times 10^{-2}$ M, respectively.
For comparison, the effects of two anticholinesterases were tested. Eserine and neostigmine did not prevent inhibition due to excess substrate, nor did they exhibit sigmoidal curves at high substrate concentration. The results with eserine and neostigmine are shown in Figs. 4 and 5, respectively. Inhibition with both inhibitors was noncompetitive. The inhibition constants for eserine and neostigmine determined from Figs. 4 and 5 were $1.2 \times 10^{-6} \text{M}$ and $4.0 \times 10^{-7} \text{M}$, respectively. The inset shows the Lineweaver-Burk plot of these data.

Atropine hydrochloride was tested as well, in order to determine if sulfate contributes to the observed effects. Fig. 6 shows the substrate saturation plot for acetylthiocholine with and without atropine hydrochloride or eserine hydrochloride. Eserine hydrochloride acted in a purely noncompetitive manner.
as indicated by Lineweaver-Burk plots (not shown). Atropine hydrochloride showed complex kinetics as did atropine sulfate.

A plot of enzyme activity as a function of increasing concentration of atropine sulfate and three different concentrations of substrate is shown in Fig. 7. In the presence of saturating concentrations of substrate (acetylthiocholine: \( I = 7.5 \times 10^{-4} \) M), the rate concentration curve is sigmoid, while the curve at the lowest substrate concentration (acetylthiocholine: \( I = 7.5 \times 10^{-5} \) M) decreases monotonically. At very high concentrations of substrate (acetylthiocholine: \( I = 7.5 \times 10^{-3} \) M), low concentrations of atropine appear to activate rather than inhibit the enzyme. This appearance is illusory, however, being merely a consequence of the manner in which the graph is plotted. At high substrate concentrations, low concentrations of atropine relieve inhibition by excess substrate rather than activate the enzyme. This is apparent since the rate with atropine never exceeds \( V_{\text{max}} \) without the inhibitor.

The Hill equation (19) can be applied to the kinetics of inhibition by atropine and can be expressed in the following form (20):

\[
\log \frac{v}{V_0 - v} = \log K - n \log I \quad \text{(for inhibitor)}
\]

where \( V_0 = v \) without inhibitor, \( I \) is the inhibitor concentration, \( K \) is a constant, and \( n \) is the order of the reaction with respect to the inhibitor. At a concentration of acetylthiocholine: \( I = 7.5 \times 10^{-4} \) M, the Hill coefficient \( n \) is 1.6. At a concentration of acetylthiocholine: \( I = 7.5 \times 10^{-3} \) M, \( n \) is 0.9. Acetylthiocholine at high concentrations, therefore, increases the apparent order of the reaction, which suggests that at least two binding sites for atropine exist per molecule of enzyme. The sigmoidicity of the middle curve (Fig. 1) may also result from the balancing of the inhibitory effect of atropine on the reaction with its release of inhibition by excess substrate.

A similar plot for eserine sulfate is shown in Fig. 8. At each substrate concentration tested, \( n < 1 \), indicating a single binding site for eserine.

**DISCUSSION**

Atropine exerts two distinct effects on acetylcholinesterase. The primary effect is competition with acetylthiocholine at the active site. The secondary effect is acceleration of substrate hydrolysis at high substrate concentration. A number of possible kinetic mechanisms may give reasonably good fits to the data reported.

It is generally believed that substrate inhibition is due to the binding of a second molecule of substrate to the enzyme to form an acetyl-enzyme-substrate complex, \( EAS \) (21–23). The decacetylation of \( EAS \) is slower than that of the acetyl-enzyme, \( EA \) (24). Atropine may combine with \( EA \) to form a ternary complex \( EAI \) (I represents the inhibitor, atropine) which may be decacylated faster than \( EAS \). This mechanism may account for the acceleration of the reaction velocity at high substrate concentrations.

The above scheme is a one-site mechanism in which the acetyl-enzyme-atropine intermediate is formed at the active site. Using CPK Atomic Models (Ealing Corporation, Cambridge, Massachusetts), we were able to determine that the bond distance between the N-methyl and the phenyl group of atropine is approximately 7 Å. The anionic site is 2.5 to 5.0 Å away from the esteratic site (25). If atropine binds to the anionic site, it must assume an orientation away from the esteratic site to activate the enzyme, otherwise it would interfere with the deacetylation mechanism which would result in a decrease in the reaction rate.

An alternate mechanism is that atropine protects the enzyme against substrate inhibition rather than accelerates hydrolysis. If this is the correct mechanism it suggests that at least two distinct binding sites must exist, i.e. a regulatory site in addition to the active site.

We propose the following mechanism which fits the kinetic data. Assume a model described by Monod et al. (20), that the R state binds the substrate and the T state binds the inhibitor. According to this model, at saturating concentrations of substrate the substrate binds to the regulatory site and converts the enzyme to the T state, which has reduced catalytic activity, and also binds the inhibitor. At high concentrations of substrate, atropine competes with the substrate at the regulatory site and prevents inhibition due to excess substrate. This hypothesis is in accord with earlier findings that at least two distinct binding sites exist on the enzyme surface for substrate and inhibitors (1–9) and suggests that inhibition of acetylcholinesterase by excess substrate may be due to an allosteric mechanism.

**REFERENCES**

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