The Inhibition of Acetylcholinesterase by 2-Pyridinealdoxime Methyl Halide

L. A. MOUNTER AND R. I. ELLIN

Weapons Development and Evaluation Laboratory, Naval Weapons Laboratory, Dahlgren, Virginia 22448, and Medical Research Laboratory, Edgewood Arsenal, Maryland 21040

(Received July 11, 1967, and in revised form June 10, 1968)

SUMMARY

The effect of varying concentrations of 2-pyridinealdoxime methyl chloride (2-PAM) on the hydrolysis of acetyl-β-methylcholine and acetylcholine by electric eel cholinesterase has been studied. With the former substrate the effects are predominantly competitive, but there are noncompetitive contributions. The dissociation constant of the enzyme-inhibitor complex has been evaluated, together with other kinetic constants. The relative magnitudes of these constants determine the kinetic behavior of the enzymic hydrolysis. Inhibition by 2-PAM chloride and other quaternary compounds may be significant in the interpretation of pharmacological and biochemical experiments.

INTRODUCTION

An important therapeutic agent in the treatment of organophosphate intoxication is 2-pyridinealdoxime methyl chloride (Pralidoxime chloride). This and related compounds can reactivate inhibited cholinesterase by nucleophilic displacement of the phosphorylating group from the active site of the enzyme (1). In addition to their capacity to reactivate inhibited enzymes, 2-PAM[1] and its congeners have direct pharmacological actions on tissues. The quaternary oximes are themselves inhibitors of cholinesterases by virtue of their high affinity for these enzymes; in low concentrations they sensitize the frog rectus abdominis muscle to acetylcholine by inhibiting its hydrolysis, whereas at high concentrations they antagonize acetylcholine.

The inhibitory action of 2-PAM is not unexpected. Yet the nature and mechanism of the inhibition may be complex, as the hydrolysis of acetylcholine by acetylcholinesterases does not follow simple Michaelis-Menten kinetics but is inhibited by high substrate concentrations (2). Friess and McCarville (3, 4) and Friess and Baldridge (5, 6) have shown that several cyclic tertiary and quaternary amines manifest a mixed form of competitive and noncompetitive inhibition of acetylcholinesterase. The often accepted competitive nature of eserine inhibition has also recently been questioned (7).

Unlike the hydrolysis of acetylcholine, acetyl-β-methylcholine (Mecholyl) at high concentrations does not show significant autoinhibition when hydrolyzed by acetylcholinesterase. Simple Michaelis-Menten kinetics is obeyed. A comparative study of 2-PAM inhibition of the enzymic hydrolysis of two substrates might provide additional parameters for the evaluation of the kinetic behavior. The present report describes a study of the effect of 2-PAM chloride on choline ester hydrolysis, together with an analysis of the results.

MATERIALS AND METHODS

Enzyme. The enzyme was acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from the electric eel, obtained from Sigma Chemical Company.

[1] The abbreviation used is: 2-PAM, 2-pyridinealdoxime methyl chloride.

Substrates. Acetylcholine bromide and acetyl-β-methylcholine chloride were obtained from Eastman Organic Chemicals. 2-PAM chloride, obtained from Aldrich Chemical Company, was repurified by recrystallization (8). All other reagents were of analytical grade.

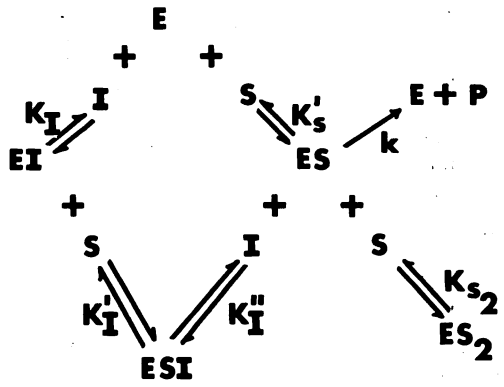
Enzyme assays. Enzyme activity was measured with a Radiometer Titrimeter at pH 7.4 without addition of buffer except for that present in the enzyme solution. Determinations were made in a microvessel (initial total volume, 2.0 ml) thermostated to 25°. The titrating agent was 0.01 N NaOH, standardized daily.

The substrate and inhibitor were diluted to a final volume of 2 ml and adjusted to pH 7.4. A baseline was run to obtain extrapolation of nonenzymic hydrolysis. The enzyme in 10^{-3} M Tris-KCl buffer (pH 7.4) containing gelatin as a stabilizing agent (9) was added at zero time, and the rate of NaOH consumption with time was recorded. At low substrate concentrations, the response was nonlinear because of decreasing substrate concentration; at higher concentrations, a linear response was obtained. Initial rates of hydrolyses were extrapolated by drawing the tangent to the curve at zero time. All results were corrected for nonenzymic hydrolysis. Results recorded were the averages of duplicate determinations. Under the experimental conditions used, it was confirmed that the rates of hydrolysis measured were directly proportional to the enzyme concentration used.

THEORETICAL

In any system an inhibitor may affect both velocity (v) and the Michaelis constant (K_s), giving a mixture of competitive and noncompetitive effects. Mixed inhibition is often neglected when the data approximately fit the special cases. More generalized inhibition equations have been developed by Hearon *et al.* (10) and Friedenwald and Maengwyn-Davis (11). The various types of inhibition can be distinguished graphically, particularly by Lineweaver-Burk (12) plots.

In the context of the present experiments



SCHEME 1

the reactions to be considered can be represented as shown in Scheme 1. The total enzyme (E_t) is given by

$$[E_t] = [E] + [ES] + [ES_2] + [EI] + [EIS]. \quad (1)$$

By applying the mass action law to the possible dissociations, the following equation may be derived:

$$\frac{V_{max}}{v} = \frac{E_t}{ES} = 1 + \frac{K_s}{[S]} + \frac{[S]}{K_{s_2}} + \frac{[I]}{[S]} \cdot \frac{K_s}{K_I} + \frac{[I]}{\alpha[K_I]}, \quad (2)$$

where V_{max} is the maximum velocity in the absence of inhibitor, v is the observed velocity at the inhibitor concentration $[I]$, and K_I is the dissociation constant of the enzyme-inhibitor complex. K'_s and the velocity constant k combine to give the Michaelis constant, K_s. In Scheme 1, if the procedure of Friedenwald and Maengwyn-Davis is applied (11), K'_I and K''_I may be eliminated and characterized by the constant α, which has a value of infinity for pure competitive inhibition and 1 for noncompetitive inhibition. Mixed inhibition gives an intermediate value, which can be determined by graphical solution (11).

One equation in Scheme 1 represents the combination of enzyme-substrate complex (ES) with excess substrate to give the inactive complex (ES_2). When this effect is significant, the rate of enzymic hydrolysis passes through a maximum and a plot of velocity against log [S] gives a symmetri-

cal bell-shaped curve. The values for K_s and K_{s_2} can be obtained by analyzing the effects of high and low substrate concentrations on the rate by the procedure of Wilson and Bergmann (13).

The hydrolysis of acetyl-β-methylcholine by acetylcholinesterase shows little or no autoinhibition. It obeys simple Michaelis-Menten kinetics and the formation of an ES_2 complex may be neglected.

The Michaelis constant (K_s) and the maximum velocity (V_{max}) must be determined for substitution in Eq. 2 above. When autoinhibition occurs, K_{s_2} must also be determined. Evaluation of K_s and V_{max} is frequently carried out by plotting $1/v$ against $1/[S]$, resulting in a linear transform of Eq. 2 whose slope is $1/(V_{max}K_s)$ and intercept is V_{max} (12), or by the method of Eadie (14), in which v is plotted against $v/[S]$. When reactions are studied over a wide range of concentrations, graphical methods of solutions are unsatisfactory, for undue weights may be contributed by extreme values of the transformed variables.

Mounter and Turner (15) have fitted data to the Michaelis-Menten equation by a weighed reiterative least squares procedure. The method has been used in the present study utilizing a FORTRAN program for the IBM 7030 computer. The procedure is almost identical with that of Hanson, Ling, and Havir (16).

RESULTS

The experimental curves for the rates of hydrolysis of acetylcholine chloride and of acetyl-β-methylcholine chloride in the absence and presence of varying concentrations of 2-PAM chloride are presented in Figs. 1 and 2. 2-PAM chloride inhibits the hydrolysis of both substrates; with acetylcholine there is a marked influence on both the rate of hydrolysis and also on the shape of the bell-shaped velocity-substrate concentration curve. At a high 2-PAM chloride concentration, the autoinhibition by excess substrate is very much decreased and shifted to the region of high substrate concentration. In this system it becomes impossible to define the extent of inhibition in terms of a simple percentage at a fixed

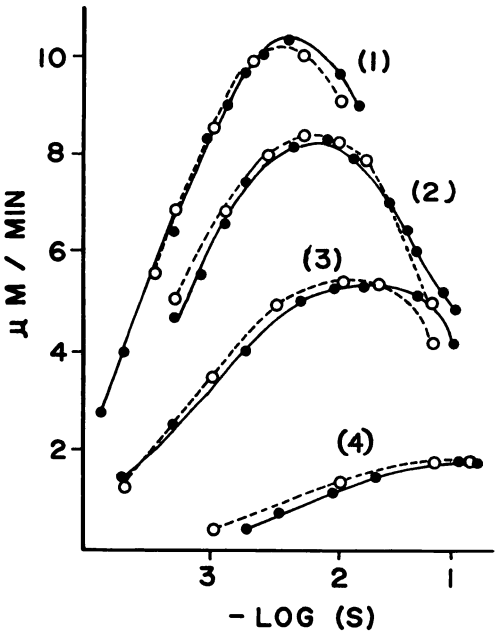


FIG. 1. *Effect of 2-PAM on enzymic hydrolysis of acetylcholine*

Curve *1*, no 2-PAM; curve *2*, 10^{-4} M 2-PAM; curve *3*, 10^{-3} M 2-PAM; curve *4*, 10^{-2} M 2-PAM. Dashed lines were calculated from Eq. 2 with the use of values from Table 1. Units of velocity are micromoles per minute.

inhibitor concentration, for the value obtained is very dependent on substrate concentration.

More simple inhibition kinetics is observed with acetyl-β-methylcholine as the substrate. It is possible to evaluate the constants which define the system in Eq. 1 from the data obtained. The procedure adopted is as follows:

Using the experimentally determined values of the enzymic rate with the two substrates and in the presence of varying concentrations of 2-PAM chloride, values of V_{max} and the apparent value of K_s are computed by the weighting procedure of Mounter and Turner (15). In the case of acetylcholine, the data points used were restricted to those observed to follow approximately linear $1/v$ against $1/[S]$ plots (17). Data from the descending (autoinhibition) portion of the $v/[S]$ curve (Fig. 1) in the absence of 2-PAM chloride were used to calculate K_{s_2} according to the

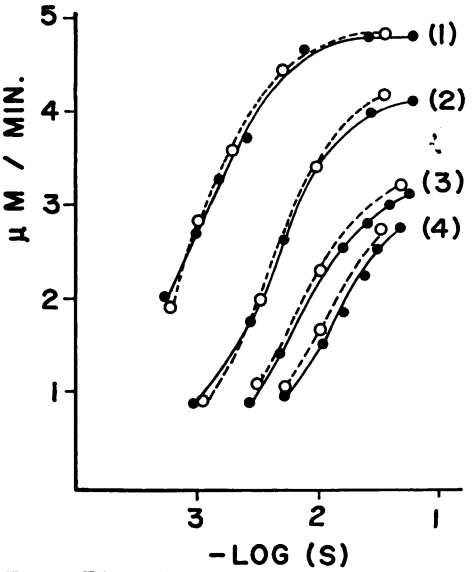


FIG. 2. *Effect of 2-PAM on enzymic hydrolysis of acetyl-β-methylcholine*

Curve *1*, no 2-PAM; curve *2*, 10^{-4} M 2-PAM; curve *3*, 2×10^{-4} M 2-PAM; curve *4*, 4×10^{-4} M 2-PAM. Dashed lines were calculated from Eq. 2 with the use of values from Table 1. Units of velocity are micromoles per minute.

procedure of Wilson and Bergmann (13). In the statistical calculation not more than two reiterations were required to obtain V_{max} and K_s, and these values were used to define the Michaelis-Menten kinetics for other forms of transposition. Plots were made of $1/v$ against $1/[S]$ for each series of data with increasing 2-PAM chloride concentration, and also of $1/v$ against $[I]$ for a series of substrate concentrations. It was observed that both inhibitions were of a "mixed" type (11, 18). With acetyl-β-methylcholine, however, the value of the constant α was high (> 50), so that the dominant factor was competitive inhibition. The value of K_I was calculated from these data by substitution in Eq. 2. With acetylcholine as substrate, the contribution of the noncompetitive substrate was greater and the value of α obtained was considerably lower. The values for the various constants are presented in Table 1.

The values were substituted in Eq. 2, and the enzymic velocity was calculated for various inhibitor and substrate concentrations. The curves obtained by this procedure are also presented in Figs. 1 and 2 and show excellent agreement with the experimental results. This indicates that the reaction scheme of Eq. 1 affords a satisfactory interpretation of the factors involved in the enzymic reactions.

TABLE 1

Kinetic constants for choline ester hydrolysis by acetylcholinesterase in the presence of 2-PAM

Constant	Acetyl-β-methylcholine	Acetylcholine
K_s	8.37×10^{-4}	3.16×10^{-4}[a]
K_{s_2}		3.5×10^{-2}[a]
V_{max}	5.13	11.19
α	>50	9
K_I	2.1×10^{-4}	2.1×10^{-4}

[a] Compare data of Wilson (19): $K_s = 2.6 \times 10^{-4}$ and $K_{s_2} = 3 \times 10^{-2}$.

CONCLUSIONS

The data presented show that 2-PAM chloride is an inhibitor of acetylcholinesterase. This inhibition is predominantly competitive, but there are also noncompetitive effects. The effect of inhibitor on the Murray-Haldane curve for acetylcholine hydrolysis is very marked, for autoinhibition by excess substrate appears to be almost eliminated by high 2-PAM chloride concentrations. The relative magnitudes of the dissociation constants of the enzyme-substrate complexes (ES and ES_2) and the enzyme-inhibitor complex (EI) account for observed experimental results. The EI complex is considerably more stable than the ES_2 complex and hence has the predominating influence. Acetyl-β-methylcholine hydrolysis is again more readily inhibited than that of acetylcholine, because of the relative magnitudes of the substrate and inhibitor affinities for the active center of the enzyme.

It should be noted, however, that the agreement observed over the wide range of concentrations involved does not necessarily validate the suggested mechanisms of reaction. Although the concept of the formation of an inactive ES_2 complex to account for the observed autoinhibition of

acetylcholinesterase has been widely accepted and provides a reasonable approximation of experimental observations, there is no direct evidence for the existence of the postulated complex. It is quite possible that other mechanisms, such as an allosteric change (20–22) in the active center of acetylcholinesterase, might provide a more satisfactory solution.

The experimental results presented in this paper yield kinetic constants for ES and ES_2 which are in good agreement with the results of Wilson (19), and the relatively small differences observed may well be due to differences in experimental conditions. The results show that the observed extent of inhibition may be quite complex with a system that cannot be described in terms of simple Michaelis-Menten theory. The inhibition of acetylcholinesterase by 2-PAM chloride may be of significance in interpreting both pharmacological and enzymic experiments.

REFERENCES

1. I. B. Wilson and F. Bergmann, *J. Biol. Chem.* **185**, 479 (1950).
2. G. A. Alles and R. E. Hawes, *J. Biol. Chem.* **133**, 375 (1940).
3. S. L. Friess and W. J. McCarville, *J. Amer. Chem. Soc.* **76**, 1363 (1954).
4. S. L. Friess and W. J. McCarville, *J. Amer. Chem. Soc.* **76**, 2260 (1954).
5. S. L. Friess and H. D. Baldridge, *J. Amer. Chem. Soc.* **78**, 199 (1956).
6. S. L. Friess and H. D. Baldridge, *J. Amer. Chem. Soc.* **78**, 966 (1956).
7. H. H. Stein, G. J. Lewis and A. J. Glasky, *Fed. Proc.* **25**, 319 (1966).
8. R. I. Ellin, U. S. Patent **3,140,289**. July 7 (1964).
9. H. O. Michel, *J. Lab. Clin. Med.* **34** (Pt. 2), 1564 (1949).
10. J. Z. Hearon, S. A. Bernhard, S. L. Friess, J. D. Botts and M. F. Morales, *in* "The Enzymes" (P. D. Boyer, H. Lardy and K. Myrbäck, eds.), Vol. I, Chap. 2, p. 49. Academic Press, New York, 1959.
11. J. S. Friedenwald and G. D. Maengwyn-Davis, *in* "Mechanisms of Enzyme Action" (W. D. McElroy and H. B. Glass, eds.), p. 154. Johns Hopkins Press, Baltimore, 1954.
12. H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.* **56**, 658 (1930).
13. I. B. Wilson and F. Bergmann, *J. Biol. Chem.* **186**, 683 (1950).
14. G. S. Eadie, *Science* **116**, 688 (1952).
15. L. A. Mounter and M. E. Turner, *Enzymologia* **25**, 225 (1963).
16. K. R. Hanson, R. Ling and E. Havir, *Biochem. Biophys. Res. Commun.* **29**, 194 (1967).
17. K. J. Laidler, "Chemical Kinetics of Enzyme Action," Oxford University Press, Oxford, 1958.
18. M. Dixon and E. C. Webb, "Enzymes," p. 326. Academic Press, New York, 1964.
19. I. B. Wilson, *in* "Mechanisms of Enzyme Action" (W. D. McElroy and H. B. Glass, eds.), p. 642. Johns Hopkins Press, Baltimore, 1954.
20. J. Monod, J.-P. Changeux and F. Jacob, *J. Mol. Biol.* **6**, 306 (1963).
21. J. Monod, J. Wyman and J.-P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
22. J.-P. Changeux, *Mol. Pharmacol.* **2**, 369 (1966).