Structural Specificity of Substrates of Acetylcholinesterase

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(Received July 18, 1966)

SUMMARY

1(2-Acetoxyethyl)quinuclidinium iodide has been examined as a substrate of both purified acetylcholinesterase and human serum cholinesterase. Neither enzyme is capable of hydrolyzing it. The inhibition of acetylcholinesterase by the quinuclidine ester has been investigated and the K_i is reported. The stability of the ester toward acetylcholinesterase is discussed in terms of the molecular perturbation theory of drug action.

INTRODUCTION

It is now well established that the interaction between acetylcholinesterase and choline esters involves two major sites, an "esteratic site" and an "anionic site." The former is responsible for the hydrolytic activity, and the latter is considered to facilitate enzymic hydrolysis by attracting, binding, and orienting cationic substrates (1, 2). The structural requirements of the acid moiety of choline esters for substrate activity have been extensively studied (3), but far less attention has been paid to the structural specificity of the quaternary ammonium group. In a discussion on the role of the quaternary nitrogen in enzyme-substrate complex formation, Belleau and Lacasse (4) concluded that the environment of the anionic site is hydrophobic, and they predicted that bulky substituents on the nitrogen atom would induce nonspecific accommodative perturbations of the enzyme, leading to a reduction in stimulating activity of the enzyme-substrate complex. A number of reports have appeared in which the effect of replacing one methyl group of the trimethylammonium group of acetylcholine with larger groups on the rate of hydrolysis by acetylcholinesterase has been examined (5–7). Unfortunately the maximum velocity of hydrolysis of these compounds at optimum substrate concentrations has not been determined, and results reported are the velocity of hydrolysis at one molar concentration for the different substrates. However, the results indicate that small groups replacing a methyl group on the nitrogen have little effect on the compounds as substrates. Even *N*-benzylnoracetylcholine is hydrolyzed at 40% the rate of acetylcholine (5).

The effect of substituting all three methyl groups by other alkyl groups in acetylcholine has been reported by Holton and Ing (6), who found that "triethylacetylcholine" was hydrolyzed by acetylcholinesterase at approximately the same rate as acetylcholine. This finding is considered further in the discussion.

Free rotation about C—C and C—N bonds is possible in all the acetylcholine derivatives so far examined, with a consequence that the conformation of the quaternary ammonium group when it is associated with the anionic site of acetylcholinesterase cannot be known.

Quinuclidine is a tertiary amine in which the conformation of the whole system is rigid and so it was considered of interest to examine the quinuclidine analog of acetylcholine as a substrate and inhibitor of acetylcholinesterase.

MATERIALS AND METHODS

Quinuclidine. 3-Quinuclidone hydrochloride (10 g) (Aldrich Chemical Co.) was dissolved in hydrochloric acid (48 ml); zinc amalgam (25 g) prepared by the method of Martin (8) was added, and the mixture was refluxed for 24 hr. More zinc

amalgam (12.5 g) and hydrochloric acid (25 ml) were then added, and the mixture was refluxed for 20 hr. The resulting solution was cooled, filtered, made alkaline with sodium hydroxide, and steam distilled until the distillate was no longer alkaline (approximately 250 ml). The distillate was acidified with hydrochloric acid and evaporated to dryness under reduced pressure. The solid residue was dissolved in water (2 ml), solid potassium hydroxide was added, and the solution was continuously extracted with ether. The ethereal extract was dried over potassium hydroxide and filtered; the ether was removed by distillation when a solid was collected. Sublimation gave a white product, m.p. 155°, lit. (9) 156°, yield 4.4 g; 64%.

Quinuclidine hydrochloride was prepared by passing dry hydrogen chloride through an ethereal solution of quinuclidine. Recrystallized from acetone-ethanol, m.p. 322–4°, lit. (10) 323–4°.

Quinuclidine picrate recrystallized from ethanol, m.p. 275–6°, lit. (9) 275–6°.

2-Bromoethanol was prepared by the method of Thayer, Marvel and Hiers (11).

2-Bromoethylacetate. 2-Bromoethanol was acetylated by standard methods using acetyl chloride, b.p. 64–5°/24 mm.

2-Iodoethylacetate. 2-Bromoethylacetate (1 mole) was refluxed, with stirring, for 1 hr with a solution of sodium iodide in acetone (2 moles, 15%). The mixture was cooled, filtered, distilled to small bulk, and filtered. Free iodine was removed from the filtrate by shaking it with mercury and filtering. The filtrate was distilled, b.p. 90°/24 mm.

1(2-Acetoxyethyl)quinuclidinium iodide (I) was prepared by refluxing a solution of quinuclidine (2.2 g) and 2-iodoethylacetate (4.28 g) in anhydrous ethanol for 0.5 hr. The solution was cooled when the product crystallized out and was filtered. Recrystallized from ethyl methyl ketone-ethanol, m.p. 136°. $C_{11}H_{10}NO_2$ requires C, 40.6; H, 6.2; N, 4.3. Found C, 40.0; H, 6.2; N, 4.7. The compound is very hygroscopic.

Bovine erythrocyte acetylcholinesterase (Nutritional Biochemicals Corporation) was used to determine K_m and K_i values. A pooled human plasma preparation was used as the source of cholinesterase. Acetylcholine perchlorate was used as a substrate and rates of hydrolysis were determined by the pH stat method using an automatic titrator equipped with expanded scale, recorder, and syringe burette (Radiometer, Copenhagen). The final volume of the reaction mixture in jacketed glass vessels was 20 ml, except at very low substrate concentrations, when the final volume was increased to 40 ml. The reaction medium consisted of 0.1 M NaCl in water from an all-glass still, and the solution was stirred with a mechanical stirrer in a CO_2-free nitrogen atmosphere throughout. The final concentration of enzyme was 0.015 mg/ml (fresh solutions were made each day and kept at 4°C). After an incubation period of 15 min in the presence or absence of the inhibitor, the substrate was added and the recorder was started. The pH was maintained at pH 7.40 ± 0.05 at a temperature of 37° by the addition of 0.01 N NaOH. The velocity was calculated from the slope of the initial linear portion of the graph and stated as volume of NaOH/min. Conditions were such that after at least 2.5 min of hydrolysis less than 20% of the total substrate available was consumed. The rates observed are corrected for nonenzymic hydrolysis of both acetylcholine and 1-(2-acetoxyethyl)quinuclidinium iodide.

RESULTS

1-(2-Acetoxyethyl)quinuclidinium iodide (I) is not a substrate of either acetylcholinesterase or human serum cholinesterase. A wide range of ester and enzyme concentrations were used.

The inhibition constant K_i for the inhibition of acetylcholinesterase by 1-(2-acetoxyethyl)quinuclidinium iodide was determined according to Eq. 1, which is based on the equilibrium assumption and the Briggs-Haldane modification of Michaelis-Menten law (11).

$$\frac{v_0}{v} = 1 + \frac{[I]}{K_i\left(1 + \frac{[S]}{K_m}\right)} \qquad (1)$$

In this expression, v_0/v is the ratio of uninhibited to inhibited rate of hydrolysis, and K_m for the hydrolysis of acetylcholine by acetylcholinesterase was found to be 1.1 $\times 10^{-4}$ M from a Lineweaver-Burk plot (12) (duplicate determination). The equation does not apply to inhibition by excess substrate; hence, suboptimum substrate concentrations were used. If v_0/v is plotted against [I] a straight line results, whose x intercept is equal to the expression 2.

$$-K_i\left(1 + \frac{[S]}{K_m}\right) \qquad (2)$$

The effect of varying the inhibitor concentration was determined at two substrate concentrations. The results are shown in Fig. 1. Since the inhibition varies with the substrate concentration used, the inhibition is largely competitive. The K_i was found to be 2.2×10^{-4} M, and the values obtained at the two substrate concentrations agree to within 5%.

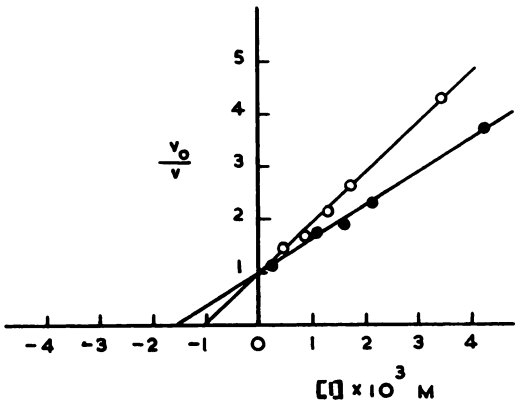


FIG. 1. *Inhibition of acetylcholinesterase by 1(2-acetoxyethyl)quinuclidinium iodide*

Key: ○, Acetylcholine concentration 4.0×10^{-4} M; ●, acetylcholine concentration 7.0×10^{-4} M; v_0, uninhibited rate of hydrolysis obtained in duplicate at each substrate concentration; v, inhibited rate.

DISCUSSION

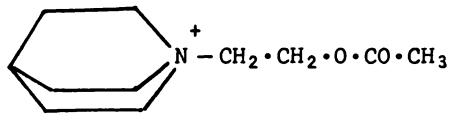
The fact that 1-(2-acetoxyethyl)quinuclidinium iodide (I) is not hydrolyzed either by acetylcholinesterase or cholinesterase is surprising, and a consideration of this fact should assist in defining more closely than previously the substrate specificity of the cholinesterases.

The hydrolysis of esters by acetylcholinesterase proceeds in four stages (a), (b), (c), (d), (13):

$$E + S \overset{(a)}{\rightleftarrows} E - S \overset{(b)}{\rightarrow} EA \overset{(d)}{\rightarrow} E + \text{acid}$$
$$\searrow^{(c)}$$
$$\text{alcohol}$$

where E represents the enzyme, S the substrate, E — S the enzyme substrate complex, and EA the acylated enzyme.

The present discussion is limited to acetate esters, with the consequence that stage (d) is common to all compounds considered. The stability of 1-(2-acetoxyethyl) quinuclidinium iodide (I) is not due to



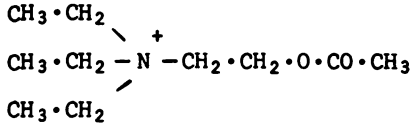
(I)

inhibition of the deacylation step (d) by either the quinuclidine ester or corresponding alcohol, since if it were it would be expected that the ester would be hydrolyzed at low concentrations, which it is not. 1-(2-Acetoxyethyl)quinuclidinium iodide (I) must, therefore, be unable to acetylate the enzyme (b) because of an unfavorable interaction in the first stage (a).

Belleau has suggested that the interaction of a compound with a receptor induces molecular perturbations in the receptor protein structure (4, 14). These can be of two types, specific and nonspecific. Specific molecular perturbations lead to the compound under examination being an agonist or substrate, and nonspecific molecular perturbations lead to the compound being an antagonist or inhibitor. On this theory 1-(2-acetoxyethyl)quinuclidinium iodide induces exclusively nonspecific molecular perturbations, and it seems to be more efficient than any other group at doing this. Very few reports have been found of other simple acetate esters which are not hydrolyzed by either of the cholinesterases.

Even non-onium esters such as ethyl acetate are hydrolyzed, and the carbon analog of acetylcholine 3,3-dimethylbutyryl acetate is hydrolyzed quite rapidly (15). In order to obtain some insight into the structural features which may be responsible for this response, it is of value to consider the activity as substrates of similar onium esters.

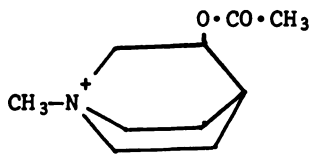
"Triethylacetylcholine" (II), which has only one carbon atom less than the quinuclidinium ester, is hydrolyzed by acetylcholinesterase (dog's caudate nucleus) al-



(II)

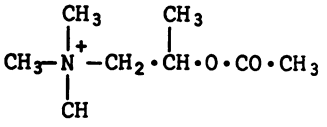
most as rapidly as acetylcholine (6). This is rather surprising since the triethylammonium group is an antagonist on most cholinergic systems, but Ariens and Simonis (16) have reported that "triethylacetylcholine" has an intrinsic activity of 1 on the jejunum of rat. A basic difference between the triethylammonium group and the quinuclidinium group is the possibility of rotation about C—N and C—C bonds with the triethylammonium group. This suggests that it is the conformation (within the range being considered) rather than the overall size of the onium group that is critical for inducing the unfavorable interaction in stage (a) above.

Another compound of interest is 3-acetoxyquinuclidine methiodide (III), which



(III)

has been reported as being a substrate for acetylcholinesterase (17). The fact that this quinuclidine ester is hydrolyzed indicates that in its interaction with the enzyme the bulk of the quinuclidine and the hydrophobic interactions associated with it are not sufficient to induce a completely unfavorable interaction in stage (a). It is possible to consider 3-acetoxyquinuclidine methiodide as a derivative of β-methylacetylcholine (IV) in which the N→O



(IV)

distance is fixed by the quinuclidine ring system. Substrates III and IV (+S isomer) are hydrolyzed at approximately the same rates.

From the considerations outlined above it appears that the particular structural features of 1-(2-acetoxyethyl)quinuclidinium which makes it stable to acetylcholinesterase and thus be an inhibitor of the enzyme is the fact that 4 carbon atoms are held rigidly beyond the nitrogen atom. A number of other acetate esters with similar structural characteristics are being examined to see whether this is a general phenomenon.

ACKNOWLEDGMENT

This investigation was supported in part by grants from the National Health and Medical Research Council of Australia. The authors wish to thank Mr. B. K. Williams for assistance with the chemical work.

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