A Novel Irreversible Inhibitor of Acetylcholinesterase Specifically Directed at the Anionic Binding Site: Structure–Activity Relationships

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SUMMARY

Among a series of *N,N*-dimethyl-2-chloro-2-phenethylamines, the prototype molecule carrying an unsubstituted phenyl ring has the unique property of irreversibly inactivating erythrocyte acetylcholinesterase when acetylcholine is the substrate. The active species responsible for this irreversible inhibition is the corresponding *N,N*-dimethyl-2-phenylaziridinium ion (DPA) which is generated spontaneously and quantitatively from the 2-chloroamine progenitor at neutral pH. The quaternary ion nature of DPA would allow for reversible addition complex formation with the anionic site of the enzyme and would precede slow alkylation of a nucleophile at that level. A significant stereoselectivity of the enzyme for the levo-isomer of DPA was demonstrated. Such optical selectivity is characteristic of the active surfaces of enzymes in general and of acetylcholinesterase in particular. When the phenyl ring carries substituents such as a 3′-hydroxy, 3′-methoxy, 3′-bromo, 4′-bromo, 4′-methoxy, or 3′,4′-dibromo, only a reversible addition complex forms. The inability of these substituted analogs of DPA to alkylate the enzyme bears no relationship to the chemical reactivity of the aziridinium rings, but suggests rather that only the unsubstituted phenyl (as in DPA) can induce in the anionic compartment the type of fit conditioning covalent bond formation. In agreement with the view that DPA interacts with the anionic site of the enzyme, the competitive inhibitor tetramethylammonium iodide was shown to be effective in retarding irreversible inactivation. Neither prolonged dialysis nor incubation with 2-pyridine-aldoxime methochloride (PAM) can reactivate the enzyme after treatment with DPA. No correlation exists between the mode of interaction of DPA and its analogs with acetylcholinesterase and their effects at the adrenergic α-receptor level. The unique properties of DPA suggest applications in the determination of the active sequence at the anionic binding site of acetylcholinesterase.

INTRODUCTION

It has long been recognized that the acetylcholinesterase (AChE)[2] catalytic surface (Acetylcholine acetyl-hydrolase, EC 3.1.1.7) includes nucleophilic sites which can for covalent bonds with acyl, phosphoryl, carbamyl, and sulfonyl groups and an anionic site which binds positively charged heteroatoms carrying alkyl substituents (1–3). When the covalent bond with an esteratic site hydrolyzes sluggishly, the kinetic behavior of the enzyme has the characteristics of irreversible inhibition (2,

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[2] Abbreviations: AChE = acetylcholinesterase; ACh = acetylcholine bromide; DPA = *N,N*-dimethyl-2-phenylaziridinium; K_i = inhibition constant; PAM = 2-pyridine-aldoxime methochloride.

3). It is of considerable significance that masking of the anionic site by suitable secondary, tertiary, or quaternary ammonium ions has a marked accelerating effect on the rate of sulfonylation and carbamylation of the esteratic site (1, 2). In contrast, such interactions with the anionic site inhibit the hydrolytic regeneration of the sulfonylated and carbamylated enzyme (1, 2). On that basis, it seems reasonable to suggest that the anionic center of AChE plays in a formal way the role of regulatory (or allosteric) site with respect to the efficiency of the esteratic compartment when the circumstances are favorable (4). It appears likely that the interaction of quaternary ions with physiological receptors may involve a regulatory anionic site similar in some way to that of AChE (4). Clearly, the chemical nature of the anionic compartment will have to be known before a more comprehensisve understanding of the mechanism of action of the enzyme and its receptor counterpart can be achieved. The presence of the hydroxymethyl of a serine residue at the esteratic center has been demonstrated (5, 6) whereas the participation of an imidazole ring has been inferred from pH dependence studies of the kinetic properties of the enzyme (7). The speculation has been offered that the anionic site may be furnished by a glutamyl residue (8, 9). An ultimate answer to this intriguing question should be provided when anionic-site specific irreversible inhibitors become accessible. The recent availability of the enzyme in homogeneous form (10) should facilitate labeling experiments with such inhibitors.

As a working hypothesis for the design of an irreversible inhibitor of AChE directed toward the anionic site, it was reasoned that the choice of chemically reactive potential inhibitors should be restricted to quaternary ion structures in order that a reversible addition complex specifically involving the anionic site precedes the formation of a covalent bond with the enzyme. Should the quaternary ion be inherently chemically reactive, covalent bond formation would likely be confined to the anionic compartment in contrast to the organophosphorus inhibitors which are selectively directed at the esteratic region. In principle, the ideal type of chemical species combining the features of a true quaternary ion and electrophilic reactivity should be the *N,N*-dialkylaziridinium ion (ethyleniminium ion), a chemical arrangement already known to produce irreversible inhibition of the adrenergic α-receptor when appropriately substituted by aromatic rings (11). We were encouraged further in the pursuance of this rational approach by the reported inhibitory activity of nitrogen mustards against AChE (12). It is known that 2-halogenoethylamines and their analogs are effective alkylating agents, a property which they owe to their ability to generate aziridinium ions. However, we rejected the nitrogen mustard type of structure for our practical purposes because their mechanism of inhibition of AChE appears ambiguous (12), an observation which probably reflects the marked lack of specificity associated with this class of drugs (13). This lack of specificity is not too surprising since nitrogen mustard and analogous structures serve as a rather mediocre source of effective concentrations of aziridinium ions (14), a property which suggests that at any given time, a complex mixture of chemical species must compete for the AChE surface. Moreover, the bifunctional reactivity of these drugs is an added complicating factor and renders more difficult the interpretation of any inhibition effects in terms of interactions with the active surface of the enzyme.

For our purposes, the ideal alkylating quaternary ion should form rapidly at neutral pH and in near quantitative yield (from the appropriate 2-haloalkylamine progenitor), should be relatively stable toward spontaneous hydrolysis but reactive toward nucleophiles, should allow for ready addition complex with the anionic site, and should be specific. A close approximation of these requirements is achieved, at least in principle, in the molecule of *N,N*-dimethyl-2-phenylaziridinium ion (DPA), a substance already known to act as a potent irreversible inhibitor of the adrenergic α-receptor (15, 16). The close structural analogy between this reactive quaternary ion

and the moderately potent but stable competitive inhibitor benzyltrimethylammonium ion (17) suggested that specific and reversible addition complex formation should initially characterize the interaction of DPA with the anionic site of AChE. Whether such an addition complex would subsequently allow for stable covalent bond formation with the anionic region of AChE had to await the test of experimentation. It is the purpose of this communication to report on our success in attaining this goal and to discuss some problems of structure–activity relationships in the DPA series of inhibitors.

METHODS

Synthetic procedures and materials. The structure and physical constants of the inhibitors used in this study are given in Table 1. Compounds *dl*-I, *d*-I, *l*-1, VI, and VII were prepared as described previously (16, 18). The others (II, III, IV, and V) were synthesized according to the scheme shown in Fig. 1.

N,N-dimethyl-3′-hydroxy-2-chloro-2-phenethylamine hydrochloride (III, Table 1). The required starting material *N,N*-dimethyl-2,3′-dihydroxy-2-phenethylamine was prepared from 3′-benzyloxy-2-dimethylaminoacetophenone (19) by heating with 10% aqueous hydrochloric acid for 3 hr followed by hydrogenation over 10% Pd/C at atmospheric pressure in 70% aqueous ethanol. After absorption of hydrogen had ceased, the catalyst was removed and the filtrate evaporated to dryness to give *N,N*-dimethyl-2,3′-dihydroxyacetophenone hydrochloride (20) as a viscous oil in near quantitative yield. The product was treated at 0° with a slight excess (5%) of thionyl chloride in dry chloroform. After it had stood 3 hr at room temperature, the mixture was evaporated to dryness *in vacuo* and the residue was recrystallized to constant melting point (m.p.) from ethanol-ether. The physical constants are given in Table 1 (compound III).

TABLE 1

Structure and physical constants of substituted N,N-dimethyl-2-halo-2-phenethylamines

$$R\text{—}\{C_6H_4CH(X)\text{—}CH_2\text{—}N(CH_3)_2\cdot HX$$

Compound No.	R	X	M.p.	Analysis[a] Calculated C	H	N	X	Found C	H	N	X
dl-I[b]	H	Cl	—	—	—	—	—	—	—	—	—
d-I[b]	H	Cl	—	—	—	—	—	—	—	—	—
l-I[b]	H	Cl	—	—	—	—	—	—	—	—	—
II	3′-OCH_3	Cl	187–9°	52.81	6.85	5.60	28.35	52.65	6.96	5.31	28.07
III	3′-OH	Cl	178–180°	50.86	6.40	5.93	30.03	50.52	6.55	5.70	29.88
IV	3′-Br	Cl	196-8°	40.16	4.72	4.68	23.17(Cl) 26.72(Br)	40.05	4.68	4.51	23.83(Cl) 26.80(Br)
V	4′-OCH_3	Cl	139–142°	52.81	6.85	5.60	28.35	52.80	6.89	5.78	28.38
VI	4′-Br	Br	180–1°	30.96	3.64	3.61	61.80	30.76	3.59	3.49	61.43
VII	3′, 4′-Br_2	Br	181-2°	25.71	2.81	3.00	68.47	25.52	2.86	3.02	68.67

[a] Performed by the Microanalytical Section, Kowa Co., Tokyo.
[b] Prepared as reported previously (16).

N,N-Dimethyl-2-chloro-3′-methoxy-2-phenethylamine hydrochloride (II, Table 1). The intermediate 3′-methoxy-2-dimethylaminoacetophenone was prepared according to the literature (19). It was re-

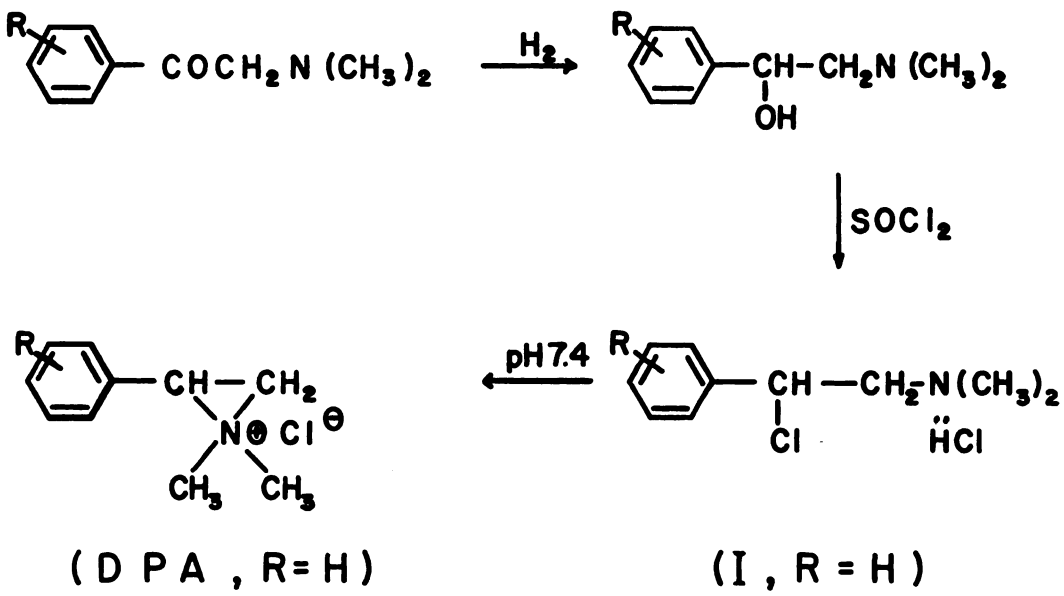

FIG. 1. *Synthesis of compounds II, III, IV, and V.*

duced with excess sodium borohydride in 20% aqueous methanol at 0° to give *N,N*-dimethyl-2-hydroxy-3′-methoxy-2-phenethylamine in 86% yield. The product was purified as the crystalline hydrochloride, m.p. 118–120° [reported: 120–2° (19)]. This material was treated with thionyl chloride in dry chloroform according to a standard procedure (21) and the product recrystallized to constant m.p. from 2-propanol. The physical constants are given in Table 1 (compound II).

N,N-Dimethyl-2-chloro-4′-methoxy-2-phenethylamine hydrochloride (V, Table 1). The starting material 4′-methoxy-2-dimethylaminoacetophenone was prepared according to the literature (18) and reduced with sodium borohydride as in the above example. The resulting *N,N*-dimethyl-2-hydroxy-4′-methoxy-2-phenethylamine (89% yield) was purified as the hydrochloride salt, m.p. 140–3° (ethanol-acetone).

Analysis. Calculated for $C_{11}H_{18}NO_2Cl$:
C, 57.01; H, 7.83; N, 6.05
Found:
C, 57.02; H, 7.89; N, 5.92

This product was converted to the 2-chloro-analog by treatment for 3 hr with a 5% excess of thionyl chloride in dry chloroform at 5–10°. The product was recrystallized to constant m.p. from methanol-ether (compound V, Table 1).

N,N-Dimethyl-2-chloro-3′-bromo-2-phenethylamine hydrochloride (VI, Table 1). Starting from the known 2,3′-dibromoacetophenone (22), 2-dimethylamino-3′-bromoacetophenone was obtained by treatment with excess dimethylamine in benzene at room temperature for 15 hr. The mixture was washed with water, and the upper phase was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was distilled *in vacuo* to give an oil, b.p. 103–9°/0.6 mm. It gave a crystalline hydrochloride, m.p. 174–6° (ethanol).

Analysis. Calculated for $C_{10}H_{13}ONBrCl$:
C, 43.08; H, 4.66; N, 5.02
Found:
C, 43.28; H, 4.53; N, 5.14

This aminoketone was reduced with sodium borohydride by the procedure described above to give *N,N*-dimethyl-2-hydroxy-3′-bromo-2-phenethylamine, b.p. 114°/0.25 mm (80% yield). The oily product was used as such in the next step, which consisted in treating it with thionyl chloride (5% excess) in dry chloroform for 1 hr at 5°

followed by heating under reflux for 30 min. The product was recrystallized to constant m.p. from ethanol–ether. The physical constants are given in Table 1 (compound VI).

Enzyme activity and inhibition. Bovine erythrocyte acetylcholinesterase (Nutritional Biochemicals Corp.) was diluted with double-distilled water made 0.04 M in $MgCl_2$ and 0.05 M in NaCl. Dilution was adjusted so that the enzyme concentration (as determined by direct assay against ACh bromide) was equivalent to 100 units per milliliter of stock solution. The rate of ACh bromide hydrolysis was followed with a pH-stat instrument (Copenhagen Radiometer) equipped with a microsyringe attachment and an Ole Dich recorder. All kinetic measurements were carried out at $25 \pm 0.1°$ and pH 7.4 using 4 ml of stock solution in a final volume of 25 ml of salt solution. A carbon dioxide-free nitrogen atmosphere was maintained throughout the measurements.

The inhibition constants K_i for all the inhibitors were calculated from Lineweaver-Burk plots constructed from the results of four different concentrations of inhibitor against a concentration of 4×10^{-4} M in ACh bromide. The K_m value for the latter with this enzyme preparation was $3.8 \pm 0.3 \times 10^{-4}$ M. In order to obtain relative but meaningful apparent K_i values, a fixed preincubation time of 3 min with each inhibitor was adopted prior to assay of the enzyme. As a reference standard for inhibition, choline chloride was compared and found to have a K_i of 4.5×10^{-4} M [for the eel enzyme the reported value is 4.5×10^{-4} M (23)].

In the case of irreversible inhibition, the time course of the reaction was followed by allowing aliquots of the enzyme stock solution containing a known quantity of inhibitor to stand at $25 \pm 0.1°$ and pH 7.4 and assaying for enzyme activity at fixed time intervals. The stock solution served as control.

Aziridinium ion formation and hydrolysis. The rate of formation of *N,N*-dimethyl-2-phenylaziridinium ions (DPA) at pH 7–7.4 in aqueous solution at 23° from *N,N*-dimethyl-2-chloro-2-phenethylamines has been reported previously (18). Cyclization of I and related compounds to the aziridinium ions is complete in less than 1 min so that a near stoichiometric amount of cyclic ions is initially formed at pH 7.4. The rate of hydrolysis of DPA at $25 \pm 0.1°$ and pH 7.4 in aqueous acetone (1:1.4 v/v) containing $MgCl_2$ (0.04 M) and NaCl (0.05 M) was followed by the thiosulfate method (21). The disappearance of DPA follows first-order kinetics (18) with a rate constant[3] $k = 7.4 \times 10^{-3}$ min^{-1}. It was previously shown (16) that the sole product of hydrolysis is the corresponding alcohol *N,N*-dimethyl-2-hydroxy-2-phenethylamine. Qualitative experiments showed that all the aziridinium ion inhibitors used in this work undergo near complete hydrolysis after about 8 hr under the above conditions. No effort was made to determine the individual rate constants because of their irrelevance in all the cases of competitive behavior (see below).

Reactivation experiments. In the unique case of irreversible inhibition by DPA (see below), the inhibited enzyme was dialyzed against distilled water at 20° for 21 hr and the $MgCl_2$ and NaCl concentrations readjusted prior to assaying. Untreated stock solution of enzyme served as control. The inhibited enzyme was incubated with PAM (Aldrich Chemical Co.) at a final concentration of 1×10^{-4} M at $25 \pm 0.1°$ and pH 7.4 for 1 and 6 hr, respectively, followed by dialysis for 21 hr at 20°. The salt concentration was readjusted, and the solutions were assayed for enzyme activity. The uninhibited enzyme was carried through the same procedures as a control.

Protection experiments. The stock solution of enzyme was made 1×10^{-2} M in twice recrystallized tetramethylammonium iodide, and aliquots were incubated at $25 \pm 0.1°$ and pH 7.4 with increasing concentrations of DPA. After an incubation time of 5 min, each aliquot was assayed for enzyme activity. As control, DPA was omitted from the reaction medium.

[3] The rate constant is nearly halved in pure water as the solvent (Dr. R. McIvor, private communication).

RESULTS

All of the compounds described in Table 1 with the exception of DPA (*dl*-I, *d*-I, and *l*-I) were found to act as competitive inhibitors of AChE. This conclusion is based on the appearance of Lineweaver-Burk plots (24) such as the typical ones shown in Figs. 2–4. After a standard preincubation they reveal that the enzyme interacts stereoselectively with 1-DPA by a factor of about 6.2 relative to the *d*-isomer. With regard to the competitive inhibitors, it is of interest to note that although the presence of a meta-OH (III) or meta-Br (IV) increases affinity significantly over that of choline, the irreversibility characteristics

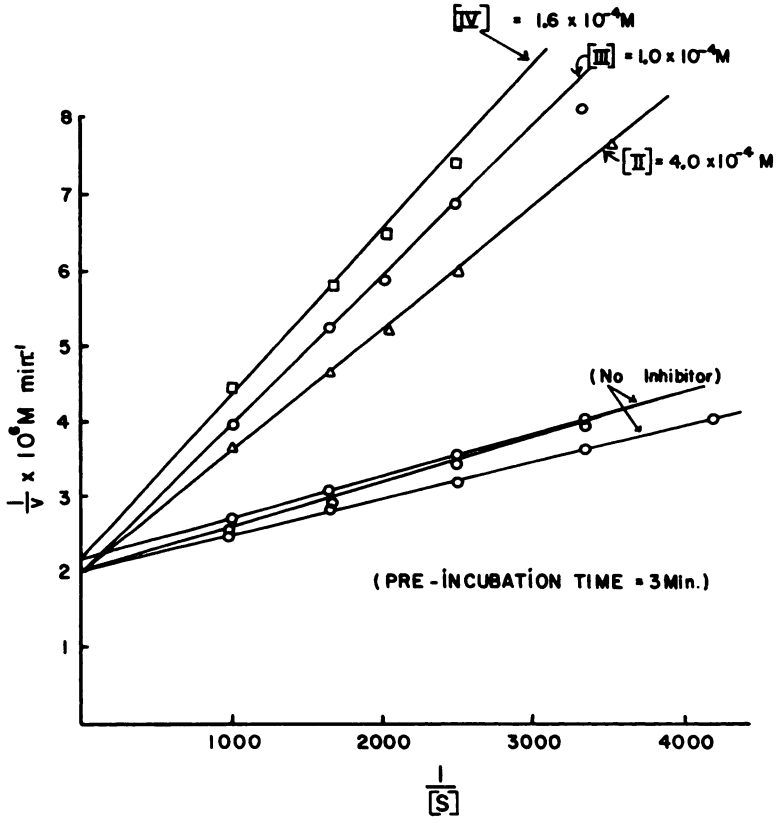

FIG. 2. *Lineweaver-Burk plots for inhibition of erythrocyte AChE (ACh bromide as substrate) by compounds II, III and IV (Table 1)*

time of 3 min, it can be clearly seen (Fig. 3) that only DPA produces a marked inhibition of the noncompetitive type. The *apparent* K_i and some I_{50} values for the entire series are assembled in Table 2. The I_{50} values for *dl*-DPA, and its optically active forms obviously cannot be compared with any of the other analogs since the respective mechanisms of inhibition are dissimilar. However, the relative I_{50} values for *d*- and *l*-DPA are informative because of DPA are nevertheless abolished by these substituents. In the case of the para-methoxy analog V, hydrolysis of the generated aziridinium ion is so rapid that preincubation with the enzyme 1 hr abolishes all inhibitory activity, thus confirming that the active species mainly responsible for inhibition are the aziridinium ions. The hydrolysis product of DPA (16, 18) (*N,N*-dimethyl-2-hydroxy-2-phenethylamine) is a weak competitive inhibitor of the enzyme

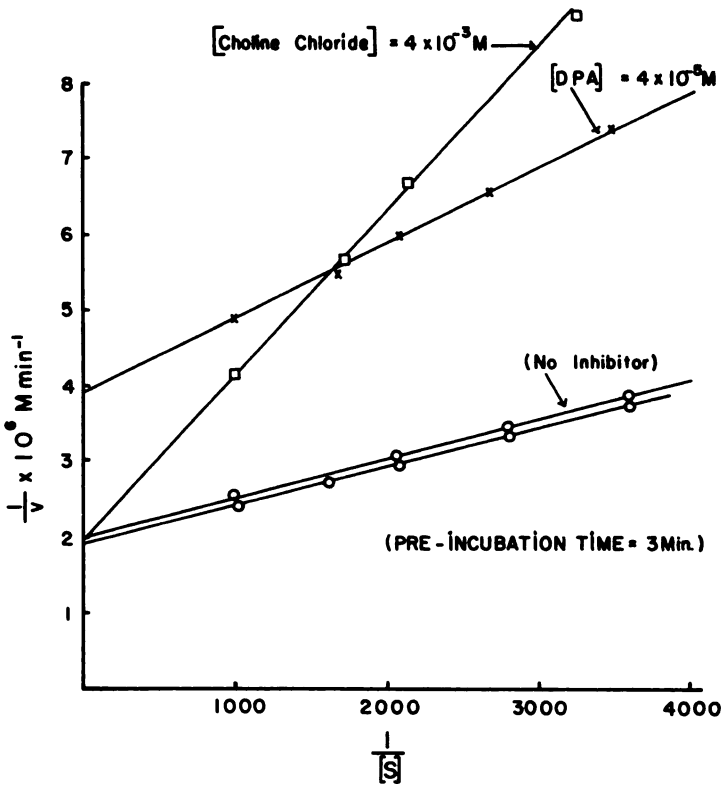

FIG. 3. *Lineweaver-Burk plots for inhibition of erythrocyte AChE by DPA and choline chloride*

TABLE 2

Inhibition constants and I_{50} values for substituted N,N-dimethyl-2-phenylaziridinium halides against bovine erythrocyte AChE

Compound No.	I_{50}(M)	$K_i \times 10^5$ M	Nature of inhibition[b]
(I) *dl*-DPA	6.0×10^{-5} [a]	0.63[a]	Irreversible
d-DPA	15.0×10^{-5} [a]	—	Irreversible
l-DPA	2.4×10^{-5} [a]	—	Irreversible
II	—	18	Competitive
III	—	4.4	Competitive
IV	—	5.8	Competitive
V	—	120	Competitive
VI	—	27	Competitive
VII	—	14	Competitive
Choline chloride	—	45	Competitive
DPA hydrolysis product	1.6×10^{-3}	—	Competitive

[a] Values obtained after a preincubation of 3 min.

[b] See Figs. 1–3.

($I_{50} = 1.6 \times 10^{-3}$ M). It follows that the noncompetitive behavior of DPA is associated with the aziridinium ring, not with the hydrolysis product.

The time-dependency of the depth of irreversible inhibition by *dl*-DPA using four different initial concentrations of inhibitor is shown in Fig. 5. After about 8

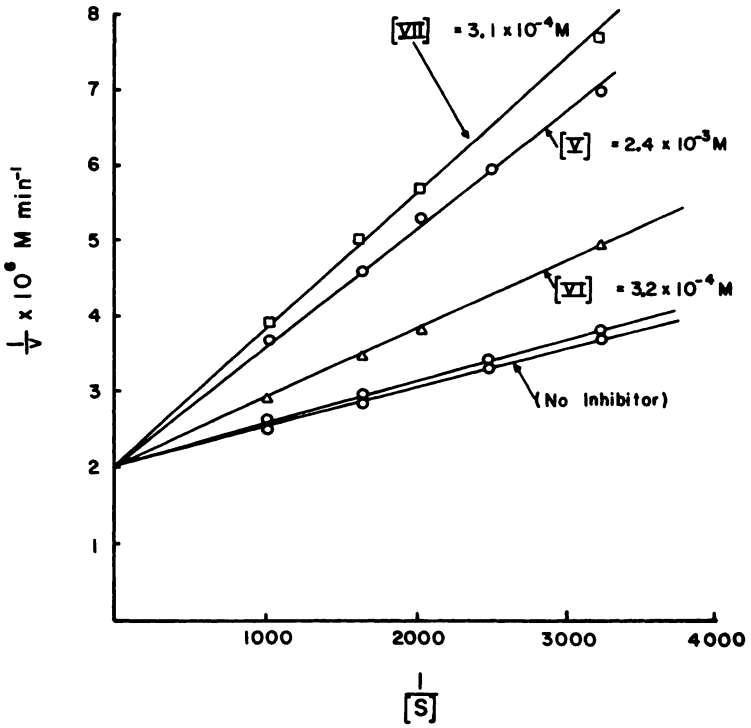

FIG. 4. *Lineweaver-Burk plots for inhibition of erythrocyte AChE by compounds V, VI, and VII (Table 1)*

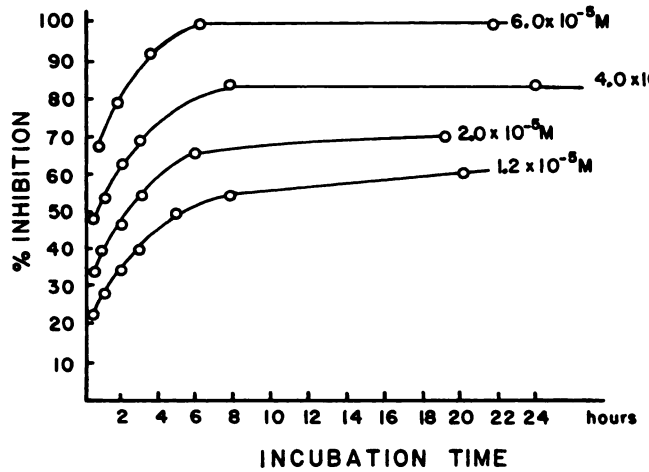

FIG. 5. *Time-course of the inhibition of erythrocyte AChE by DPA at four concentrations*

Portions of stock solution of AChE (100 units/ml) were made 1.2, 2.0, 4.0, and 6.0×10^{-5} M in DPA, respectively, and incubated at 25°. Aliquots of each solution were assayed for enzyme activity at various time intervals. The per cent inhibitions were obtained by taking the ratios of the inhibited initial velocities of ACh hydrolysis to the initial velocity of hydrolysis by the control solution. Reference conditions for 100% inhibition consisted in heating an aliquot of stock solution for 5 min at 100° prior to assaying for enzyme activity; incubation with DPA, 6×10^{-5} M for 6 hr at 25° had the same effect as the heat treatment.

hr, the percentage inhibition levels off, which corresponds to near complete hydrolysis of DPA, as can be estimated from the hydrolysis rate constant of the aziridinium ion. The irreversibility characteristics of the inhibition by DPA was established through dialysis experiments, the results of which are summarized in Table 3. It can be seen that neither dialysis alone nor incubation with PAM followed by dialysis had any effect on the depth of inhibition. This contrasts with the reactivating effect of PAM on inhibition by the organophosphorus drugs (25).

Protection of the enzyme against DPA by tetramethylammonium, a competitive inhibitor interacting specifically with the anionic site (23), could be readily demonstrated, as shown in Fig. 6. It seems possible that the presence of tetramethylammonium may repress the formation of an initially reversible complex between DPA and AChE.

TABLE 3
Effect of dialysis and incubation with PAM on DPA inhibited AChE[a]

Enzyme sample	Experimental conditions applied	Per cent inhibition
1	Dialysis 21 hr	0
2	Incubation 15 min DPA 5×10^{-5} M	70
3	Sample 2 followed by dialysis 21 hr	97
4	Sample 3 incubated with PAM 1×10^{-4} M for 1 hr[b]	100
5	Sample 3 incubated with PAM 1×10^{-4} M for 6 hr[b]	100

[a] The procedure consisted in allowing a portion of stock solution of enzyme (100 units/ml) made 5×10^{-5} M in DPA to stand for 15 min prior to assaying for enzyme activity. After 15 min, the solution was dialyzed for 21 hr at 20° (sample 3). Aliquots of the dialyzed solution were incubated with PAM prior to assaying (samples 4 and 5). Stock solution of enzyme was carried through the same procedures as a control. Reference conditions for 100% inhibition consisted in incubating the stock solution in the presence of DPA, 6×10^{-5} M for 6 hr.

[b] Similar treatment of the uninhibited enzyme with PAM showed that no loss of activity occurs.

DISCUSSION

The results clearly show that DPA inhibits irreversibly by interacting at the

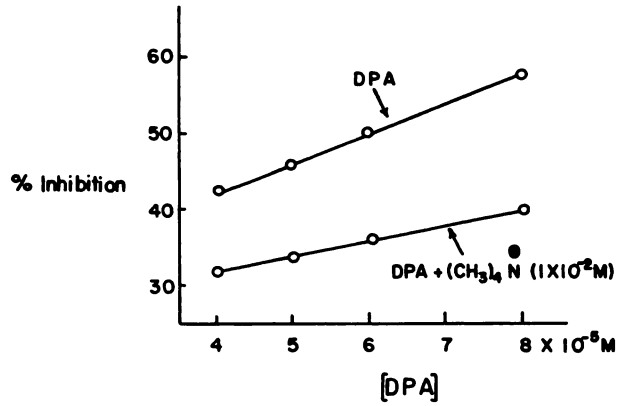

FIG. 6. *Effect of tetramethylammonium iodide on the inhibitory effect of DPA on erythrocyte AChE as a function of concentration*

A portion of stock solution of enzyme (100 units/ml) was divided into eight parts, four of which were made 1×10^{-2} M in tetramethylammonium iodide. These four portions were paired with the other four and each pair was made 4, 5, 6, and 8×10^{-5} M in DPA, respectively. After 5 min, an aliquot from each pair was assayed for enzyme activity. The per cent inhibitions were obtained from the ratios of the initial velocity of ACh hydrolysis to the initial velocity of hydrolysis by the untreated stock solution. The per cent inhibitions observed with the samples containing tetramethylammonium included the constant contribution of the latter (1×10^{-2} M being equivalent to I_{50}); this value was subtracted to give the results shown.

anionic site of AChE. That the inactivation of AChE toward its substrate ACh is the result of a specific interaction with a binding site of the active surface is evidenced by (a) the optical selectivity of the enzyme toward the *l*-isomer of DPA; (b) the effectiveness of tetramethylammonium in retarding inactivation of the enzyme by DPA; (c) the high specificity of the enzyme with regard to irreversible inactivation toward the structural features of DPA. Because of the well known chemical reactivity of aziridinium ions under physiological conditions, it is logical to conclude that the irreversible inhibition produced by DPA is the result of specific covalent bond formation with a functional group of the active surface. This conclusion is strengthened by the fact that the chemically unreactive but structurally closely related analog benzyltrimethylammonium ion produces only competitive inhibition of AChE (17). The covalent bond which develops between DPA and a nucleophilic site differs from the bond which results from attack of the esteratic site by the organophosphorus drugs (25) since PAM is ineffective in reactivating the DPA-inhibited enzyme.

The present data are insufficient for an analysis of the kinetics of covalent bond formation between DPA and AChE. A glance at the parameters of the overall kinetics of the reaction suggests that the problem should form the subject of a separate investigation. Nevertheless, a safe guess would be that the alkylation reaction follows pseudo-first order kinetics, which may explain in part the dependence on the initial concentration of DPA of the steepness of the slopes of the rates of inhibition during the early phase of the reaction. A complicating factor is that at any given time prior to total inactivation, a substantial component of competitive inhibition by DPA and its hydrolysis product is present.

The high specificity of the enzyme in the formation of a covalent bond with DPA deserves comment.[4] It is significant that substituents on the phenyl ring of DPA (Table 1) abolished irreversible inhibition without preventing competitive binding. This shows that chemical reactivity of the aziridinium ions bears no relationship to their qualitative properties at the anionic binding site level. For instance, the 4′-methoxy analog (V) of DPA displays only competitive behavior in spite of its much greater reactivity than DPA toward water. No correlation between Hammett substituent constants (26) and behavior toward the enzyme is discernible. More surprising still is the fact that the introduction of a 3′-OH as in (III) (Table 1) should contribute relatively little to affinity as well as preclude covalent bond formation with the active surface. This contrasts with the dramatic effect of a meta-OH on the affinity of phenyltrimethylammonium ion for AChE (17). On that basis, one might have expected 3′-hydroxy-DPA (generated from III) to produce a better fit on the enzyme and in this way promote covalent bond formation. Since this is not the case, one may speculate that addition complex formation with the DPA series of inhibitors may not involve the esteratic site, but only the anionic region of the enzyme. The results of Wilson *et al.* (1, 2) show in fact that certain quaternary ions (such as tetraethylammonium) can bind on the anionic site without any *positive* involvement

[4] The close structural analogy of DPA to benzyltrimethylammonium suggests that the alkylation step leading to irreversible inhibition may be preceded by reversible addition complex formation. According to the classification given by Webb (27), the process may be represented by the equation

$$E + DPA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.DPA \xrightarrow{k_2} X$$

where E is AChE and X the final product. It is clear then that $k_2 > 0$ only in the case of DPA since all its analogs act competitively ($k_2 = 0$). It follows that it is the structural and conformational properties of the complex E.DPA that would determine the magnitude of k_2; in other words, only DPA would give an addition complex that is productive with respect to covalent bond formation with AChE. The possibility also exists that the equation $E + DPA \rightarrow X$ may represent the phenomenon; however, the first interpretation has greater appeal to us.

of the esteratic site. Wilson has suggested that an accelerator site which may or may not be identical to the anionic binding site may be involved (1, 2). For these reasons, we tentatively conclude that DPA forms a covalent bond with a nucleophile that forms an integral part of the anionic compartment, and this without any involvement of the esteratic nucleophiles.[5]

The unique ability of DPA to produce a covalent bond with the anionic region may well reflect the operation of a subtle and specifically induced conformational perturbation of the anionic compartment attending addition complex formation.[4] The presence of ring substituents on DPA would serve only to induce additional perturbations that would place the key functional group acting as the nucleophile out of reach of the electrophilic carbon of the aziridinium rings. If this is the case, DPA could then be considered as a highly specific irreversible inhibitor of AChE when ACh is the substrate.

The stability at neutral pH of the covalent bond which forms between DPA and the anionic compartment suggests that a nonsolvolyzable type of bond may be involved. It would seem improbable on that basis that an ester linkage with a glutamyl residue (8, 9) constitutes the mode of attachment of DPA. Model ester derivatives of *N,N*-dimethyl-2-hydroxy-2-phenethylamine have been shown to suffer ready spontaneous hydrolysis at neutral pH (28). Similar esters derived from aziridinium ions and proteins are known to be equally sensitive toward hydrolysis (29). Functional groups like the ϵ-amino of a lysine residue, an imidazole nitrogen, a cysteine thiol, a tyrosine hydroxyl, or a serine hydroxyl are more likely to be involved in the formation of a bond with DPA because of the expectable greater stability of such bonds toward solvolysis at neutral pH. Labeling experiments using ^{14}C-DPA are in progress so as to settle this interesting question of the nature of the functional group responsible for covalent bond formation.

It is worth noting, finally, that no obvious correlation seems to exist between the inhibitory properties of the DPA series of drugs toward AChE and their effects at the adrenergic α-receptor level (30). The fact that DPA produces a transitory irreversible blockade at this level (31) (receptor regeneration taking place over a 2-hr period) strongly suggests that, in this case, an ester linkage is involved in the attachment of DPA (31). The contrasting stability of the bond between DPA and AChE supports the hypothesis that a glutamyl residue may not be involved as a site of attachment.

[5] Conclusive evidence that this is the case will be published by R. M. Heggie, R. McIvor, and J. Purdie of the Defence Research Board, Ottawa.

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