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# CYCLOPHOSPHAMIDE, 2,2-DIMETHYLAZIRIDINES AND OTHER ALKYLATING AGENTS AS INHIBITORS OF SERUM CHOLINESTERASE*

DAVID LALKA and THOMAS J. BARDOS

Departments of Biochemical Pharmacology and Medicinal Chemistry, State University of New York at Buffalo, N.Y. 14214, U.S.A.

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**Abstract**—The effects of cyclophosphamide (CTX) and of alkylating agents containing aziridine or 2,2-dimethylaziridine moieties on the procaine esterase activity of horse serum cholinesterase were investigated. The results indicated that CTX is a competitive, reversible inhibitor of the enzyme, while all the other agents studied caused irreversible inhibition. However, there was no over-all correlation between the cholinesterase inhibitory activities of these agents and their alkylating reactivities toward the model nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP). The kinetics of inhibition were consistent with the formation of a reversible enzyme–alkylating agent complex prior to the irreversible inactivation of the enzyme. In the case of the ring-C-unsubstituted aziridines (TEM, TEPA and AB-100), the inactivation process could be described by the Main equation from which a dissociation constant ($K_d$) and a reaction rate constant ($k_2$) were calculated. The 2,2-dimethylaziridines (AB-132, AB-163 and TEPA-132) readily hydrolyzed, with rapid loss of alkylating activity (vs NBP). Simultaneously, the cholinesterase inhibitory activities of AB-132 and AB-163 significantly increased, reached a maximum and then gradually decreased on further hydrolysis; in contrast, TEPA-132 showed progressive loss of inhibitory activity. These results indicate that both AB-132 and AB-163 (but not TEPA-132) hydrolyze with the formation of an unstable intermediate(s) having little or no alkylating activity but acting as a potent, irreversible cholinesterase inhibitor(s).

The cholinesterase inhibitory activity of an antineoplastic alkylating agent was first observed during the initial clinical studies of the "dual antagonist" AB-132† [1, 2]. Several patients treated with this drug exhibited a vastly increased sensitivity to succinylcholine [2–4]. Subsequent studies showed that treatment with AB-132 caused a marked decrease in the serum and red blood cell cholinesterase activity levels [4], and it appeared that some of the typical side effects of this drug could be attributed to the inhibition of cholinesterase [2]. The potentiation of succinylcholine toxicity by AB-132 and some other 2,2-dimethylaziridine derivatives was confirmed in animal experiments [2, 5]. Results of preliminary studies *in vitro* [3] with AB-132 suggested that the anticholinesterase effects of this drug were due to a hydrolysis product rather than to the drug *per se*. It was found that AB-132 undergoes a very fast hydrolytic reaction with cleavage of the 2,2-dimethylaziridine ring [1, 6]; the end products of this reaction have been isolated and identified‡ as urethane and di-(1-amino-2-methyl-2-isopropyl)phosphate (HP, Table 1); neither of these compounds has significant cholinesterase inhibitory activity. However, the nearly quantitative formation of the diester HP suggests that the mechanism of reaction involves internal P—N → P—O rearrangements (of the tertiary carbonium ions [1] initially formed upon the cleavage of each ring) which would require the formation of 5-membered cyclic intermediates; the one postulated for the rearrangement of the second aziridine moiety is shown in Table 1 (HX). Since compounds like HX are potential phosphorylating agents, it is conceivable that this intermediate could be partly responsible for the anticholinesterase effect of the drug.

More recently, similar pharmacologic effects were observed after treatment of patients with CTX [7–9]. This is particularly interesting in view of earlier studies performed in this laboratory [10] which indicated certain similarities between the mechanisms of action of CTX and AB-132. However, a number of other alkylating agents were recently reported to have anticholines-

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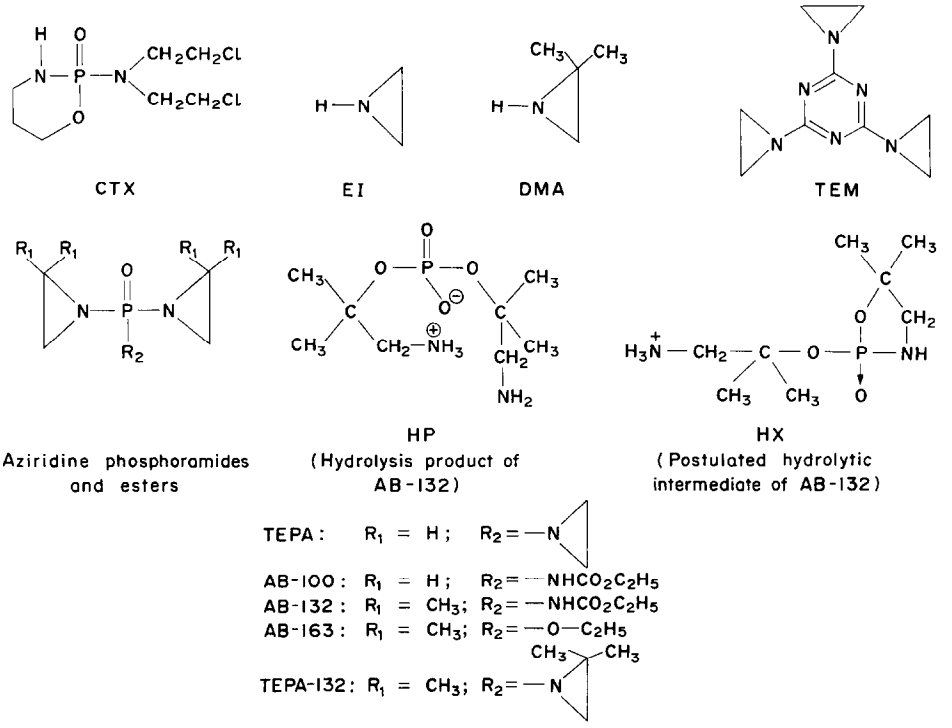
† Abbreviations: CTX, cyclophosphamide; NBP, 4-(*p*-nitrobenzyl)pyridine; AB-132, bis(2,2-dimethyl-1-aziridinyl)-phosphinyl urethane; AB-163, ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate; TEPA-132, Tris(2,2-dimethyl-1-aziridinyl)phosphine oxide; AB-100, bis(1-aziridinyl)phosphinyl urethane; TEPA, Tris(1-aziridinyl)phosphine oxide; TEM, Tris(1-aziridinyl)melamine; EI, aziridine (ethylenimine); and DMA, 2,2-dimethylaziridine.

‡ T. J. Bardos, A. K. Barua and Z. F. Chmielewicz, unpublished observations.

Table 1. Chemical structure of alkylating agents and hydrolysis products




terase activities *in vitro* [8]. Therefore, the relationship between anticholinesterase and alkylating activities seemed worthy of investigation.

The structures of the alkylating agents included in the present study are shown in Table 1.

## MATERIALS AND METHODS

The procaine esterase activity of horse serum cholinesterase was measured spectrophotometrically as the rate of decrease of absorbance at 300 nm, with time. The methodology employed was based upon previously published reports [8, 11].

### *Reagents*

Horse serum cholinesterase (EC 3.1.1.8) was a product of Sigma Chemical Co. (Type IV). Procaine hydrochloride, a product of Mallinckrodt Chemical Works, was used as substrate. Enzyme and substrate solutions were prepared fresh daily in 0·066 M $NaH_2PO_4$, pH adjusted to 7·4 with NaOH. Procaine solution of $5·6 \times 10^{-5}$ M will be termed "substrate solution," and 5·1 units/ml solutions of enzyme will be called "enzyme solution".

Cyclophosphamide was a product of Mead, Johnson & Co., bis(1-aziridinyl) phosphinyl urethan (AB-100) was obtained from the Armour Pharmaceutical Co., and ethylenimine (EI) was purchased from Dow Chemical. The sources of the other alkylating agents included in this study, and the methods used for their colorimetric determination, were previously reported [12].

### *Effects of alkylating agent–cholinesterase contact time on the magnitude of enzyme inhibition*

At zero time, 1·0 ml of a solution of the alkylating agent (or 1·0 ml of the $NaH_2PO_4$ buffer for control tubes) was added to 15·0 ml enzyme solution which had been preheated to 37°. The mixture was maintained at 37° for the duration of the experiment. At various times, duplicate 1·5-ml aliquots of each solution were withdrawn and quickly brought to 24°. Procaine solution (1·5 ml) was added to each aliquot. The initial rate of decrease of absorbance at 300 nm was measured against a blank which contained concentrations of enzyme and alkylating agent identical to the concentrations in the sample tubes. The per cent inhibition was calculated as:

$$\% \text{ inhibition} = \frac{\Delta A_c/t - \Delta A_s/t}{\Delta A_c/t} \times 100 \qquad (1)$$

where $\Delta A_c/t$ = mean change in absorbance per unit time in control cuvettes and $\Delta A_s/t$ = mean change in absorbance per unit time in sample cuvettes.

In typical experiments, procaine hydrolysis rates in the control cuvettes were about 0·08 nmole/ml/min

(see Fig. 3), and the reaction was followed for 20–40 min. During this time about 10–20 per cent of the procaine present was hydrolyzed, causing a change of absorbance of 0·035 to 0·070.

*Cholinesterase inhibition by hydrolysates of alkylating agents*

The 2,2-dimethylaziridine phosphoramides were dissolved in 15·0 ml buffer which had been preheated to 37°. These solutions were maintained at 37° throughout the experiment. At various times, 1·0-ml aliquots of the alkylating agent hydrolysate solutions were removed and added to 2·5 ml enzyme solutions which had been preheated to 37° (the concentration of the enzyme solution used in these experiments was such that it yielded the same final concentration as above). This mixture was maintained at 37° for exactly 10 min and was then quickly brought to 24°. Then 3·5 ml substrate solution was added to the enzyme–alkylating agent mixture and the rate of change of absorbance at 300 nm was measured in duplicate along with appropriate controls. Blank tubes were prepared as above. Per cent inhibition was calculated as above.

*Change of alkylating activity during hydrolysis*

The remaining alkylating activity at various times during hydrolysis was determined with the reagent 4-(*p*-nitrobenzyl)pyridine, using the previously reported procedures [12].

*Methodology for the determination of* $k_i$, $k_2$ *and* $K_d$ *from the Main equation* [13–15]

The $k_i$ (the bimolecular reaction constant) is numerically equal to $k_2/K_d$, when $K_d = k_{-1}/k_{+1}$, and the inhibitory process can be adequately described by the reaction [13]

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (EI)_{rev} \xrightarrow{k_2} (EI)_{irrev}$$

Two plotting methods have been reported for approximation of the constants $k_2$, $K_d$ and $k_i$. According to the equation

$$\frac{1}{i} = \frac{\Delta t}{2{\cdot}3\,\Delta \log v} k_i - \frac{1}{K_d} \qquad (2)$$

the plot of $1/i$ against $(\Delta t/2{\cdot}3\ \Delta \log v)$ has a slope of $k_i$, an intercept of $(-1/K_d)$ on the ordinate, and an intercept of $(1/k_2)$ on the abscissa. The term $(1/i)$ is the reciprocal of the inhibitor concentration before the addition of substrate solution, $(\Delta t)$ is the contact time (defined as the preincubation time of the inhibitor and the enzyme, from zero time until the addition of substrate), and $(\Delta \log v)$ is the logarithm of the quotient of the velocity of the enzyme reaction in the absence of inhibitor divided by the velocity of this reaction in the presence of inhibitor. This plotting method was used for TEPA and AB-100 (see Fig. 4).

The second plotting technique is a simple modification of the first [14, 15]. A plot of the rearranged equation

$$\frac{i\,\Delta t}{2{\cdot}3\,\Delta \log v} = \frac{i}{k_2} + \frac{1}{k_i} \qquad (3)$$

provides better estimates of $k_i$, $k_2$ and $K_d$ under certain experimental conditions. The slope of this plot gives $1/k_2$, the intercept of the abscissa yields $-k_2/k_i$ (i.e. $-K_d$) and the intercept on the ordinate yields $1/k_i$. The data obtained for TEM were more conveniently plotted in this manner (see Fig. 5).

*Rate of alkylation of NBP by various aziridines*

One milliliter of an acetophenone solution containing 25 per cent NBP and 5 per cent acetic acid was mixed with 1·0 ml of an acetophenone solution containing 0·45 m-equiv./liter of alkylating agents (EI, AB-100, TEM, TEPA and AB-132). The mixture was rapidly heated to 50°, and duplicate 1·0-ml aliquots were removed at various times and then rapidly cooled to 24°. *N*-phenylpiperazine (0·5 ml) was added to each aliquot to develop color, and the volume of the sample was made up to 3·0 ml with acetophenone. Absorbance at 575 nm was determined, as described previously [12].

If the solutions contain equivalent concentrations of the alkylating agents, and all assay conditions are kept constant, then the change of absorbance with time, i.e. the relative rate of alkylation, can be used as a measure of the "comparative chemical alkylating activities" of the various agents, as it was previously demonstrated [16]. It should be pointed out that, in the present paper, the comparative alkylating activities were determined for equivalent concentrations of the alkylating functional groups rather than equimolar concentrations of the compounds, and that the technique used (as described above) differs from our original method [16] in that it employs anhydrous conditions and thus eliminates the effect of competing hydrolysis [12] during the reaction with NBP. A reaction temperature of 50° was found convenient for the demonstration of significant differences between the rates of reaction of the various aziridine derivatives.

## RESULTS

Figure 1 shows the inhibitory effects of the conventional alkylating agents TEM, TEPA and AB-100 (the ring-C-unsubstituted congener of AB-132) on horse serum cholinesterase as a function of the "contact time", i.e. the length of preincubation of the alkylating agent–enzyme mixture prior to the addition of substrate. The results of similar experiments with the 2,2-dimethylaziridine derivatives (AB-132, AB-163 and TEPA-132) as well as with CTX are presented in Fig. 2. The graphs clearly show that the various aziridines cause progressive inhibition of enzyme activity as the contact time increases, while CTX produces an immediate inhibition which does not progress. It should be noted that these experiments were conducted at widely

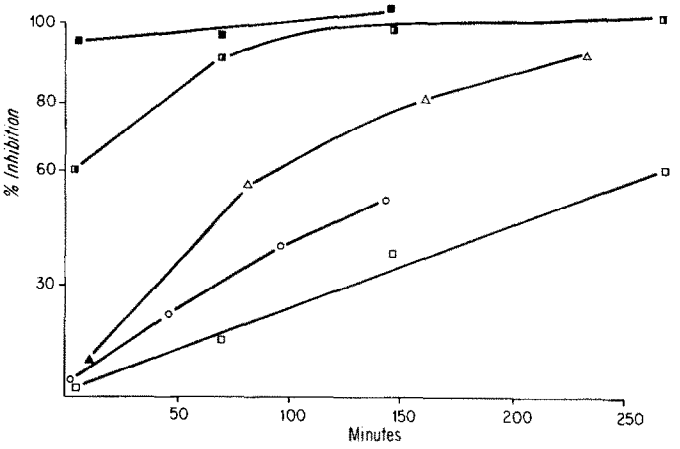



Fig. 1. Effect of alkylating agent–enzyme contact time upon inhibition of horse serum cholinesterase activity by ring-C-unsubstituted aziridine derivatives. TEPA, 5·6 mM (■); 0·56 mM (◨); and 0·056 mM (□); TEM, 0·25 mM (○); and AB-100, 2·3 mM (△).

different concentrations of the various alkylating agents; the concentration for each agent was so selected as to permit optimal convenience and accuracy of measurement. The data for TEPA are given at three different concentrations over a 100-fold range (see Fig. 1) in order to show the concentration dependence of the contact time required for maximal inhibition. The relative magnitudes of the inhibitory activities of the various agents can be judged from the $I_{50}$ data given in Table 2.

Table 2. Concentrations of alkylating agents required for 50 per cent inhibition of the procaine esterase activity of horse serum cholinesterase ($I_{50}$)

| Compound | Contact time* (min) | $I_{50}$ (mM) |
|---|---|---|
| CTX | 0† | 0·008 |
| TEM | 0 | 3·8 |
| | 10 | 1·2 |
| | 30 | 0·98 |
| | 75 | 0·44 |
| | 130 | 0·22 |
| TEPA | 0 | 0·56 |
| | 210 | 0·058 |
| AB-100 | 72 | 2·3 |
| Ethylenimine | 80 | > 13‡ |
| AB-132 | 0 | 0·34 |
| | 30 | 0·17 |
| | 70 | 0·11 |
| TEPA-132 | 8 | 1·5 |
| | 140 | 0·11 |
| AB-163 | 42 | 0·14 |
| DMA | 80 | > 14§ |

* Contact time: defined as preincubation time of the enzyme with the inhibitor, prior to the addition of the substrate.

† Inhibition by this agent is independent of contact time for at least 1000 min (see Fig. 2).

‡ No inhibition was observed after exposure of enzyme to this concentration of ethylenimine for the stated length of time.

§ Thirty-nine per cent inhibition was observed after exposure of enzyme to this concentration of 2,2-dimethylaziridine for the given length of time.

Kinetic analysis of the inhibitory action of CTX by the graphical method of Lineweaver and Burk [17] shows that this inhibition is of the competitive type, with a $K_i$ of $4{\cdot}9 \times 10^{-6}$ M (Fig. 3).

Analysis of the data for the various aziridines is somewhat more complex. It has been stated that the bimolecular reaction constant ($k_i$) is the most reliable criterion for the measurement of the inhibitory potency of a reactive compound [13]. The inhibition caused by the ring-C-unsubstituted agents (TEM, TEPA and AB-100) can be viewed as the combined

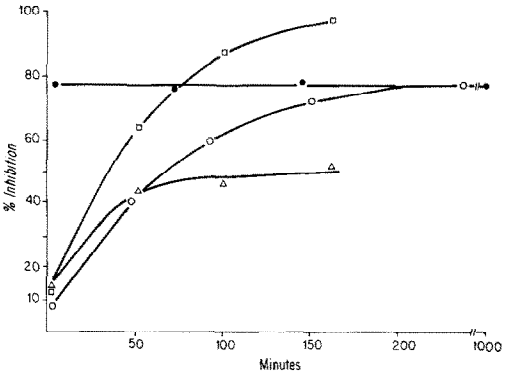



Fig. 2. Effect of alkylating agent–enzyme contact time upon inhibition of horse serum cholinesterase activity by 2,2-dimethylaziridine derivatives and CTX. Initial concentrations were: AB-163, 0·14 mM (□); AB-132 0·11 mM (○); TEPA-132, 0·12 mM (△); and CTX, 0·029 mM (●).

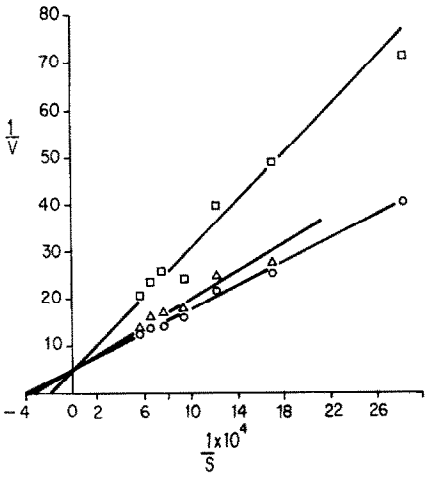



Fig. 3. Lineweaver–Burk plot of the inhibitory effect of CTX, 0·0058 mM (□) and 0·0012 mM (△), upon the rate of hydrolysis of procaine by horse serum cholinesterase; the latter, without inhibitor (control) is represented by (○). V = nmoles/ml/min hydrolyzed, and S = molar concentration of substrate. The intercepts of the lines corresponding to the two CTX concentrations yielded $K_i$ values of 4·8 and 5·0 × $10^{-6}$ M, respectively, i.e. an average $K_i$ of 4·9 × $10^{-6}$ M for CTX, using the $K_m$ value of 2·5 × $10^{-5}$ M for procaine obtained from the intercept of the control.

effects of competitive binding and irreversible alkylation. In the case of the more selective organophosphate inhibitors of cholinesterase, the Main equation [13–

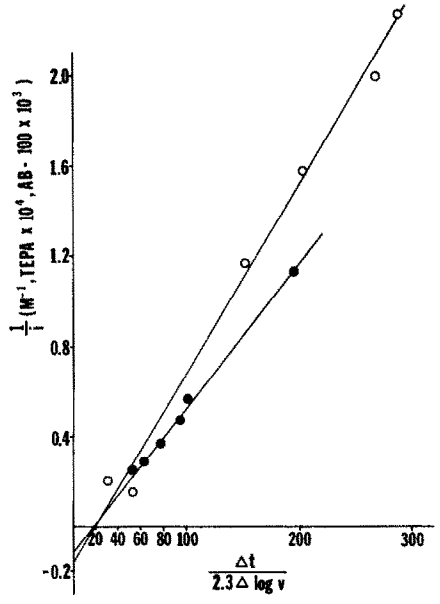



Fig. 4. Main plots of the data for the inhibition of horse serum cholinesterase by AB-100 (●) and for TEPA (○). The constants obtained from the slopes and intercepts of these plots (see Materials and Methods) are given in Table 3.

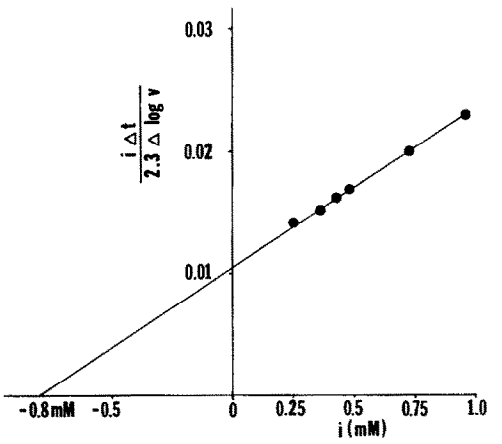



Fig. 5. Main plot of the data for the inhibition of horse serum cholinesterase by TEM (●), using the second plotting technique described under Materials and Methods.

15] appears to have served well for the separation of the reversible and irreversible components of the overall inhibitory effect ($K_d$ and $k_2$, respectively; see Materials and Methods). The plots obtained by this treatment of the data for the alkylating agents are presented in Figs. 4 and 5. The corresponding values for the constants $k_2$, $K_d$ and $k_i$ are given in Table 3.

Analysis of the inhibitory effects of the 2,2-dimethylaziridine derivatives (AB-132, AB-163 and TEPA-132) could not be accomplished in this manner, because these compounds undergo rapid hydrolysis [12], which changes the concentration (and composition) of the inhibitor(s) during the assay. The effect of prior hydrolysis upon the cholinesterase inhibitory activity of the 2,2-dimethylaziridine derivatives is shown in Fig. 6.

It can be seen that the inhibitory activities of AB-163 and AB-132 reach a maximum after about 70 min of hydrolysis at 37°. This strongly suggests the formation of a transient intermediate(s) with high anticholinesterase activity. On the other hand, the total alkylating activity, determined by the NBP assay [12], shows a rapid decrease during the same period of hydrolysis (Fig. 6), indicating that the cholinesterase inhibitory activity is probably unrelated to the alkylating activity. DMA has previously been reported to be a minor hydrolytic intermediate of at least some 2,2-dimethylaziridine phosphoramides [12]. This compound is a reactive alkylating agent, but it is essentially inactive as an inhibitor of the cholinesterase (see Table 2). In contrast to AB-132 and AB-163, TEPA-132 hydrolyzes with progressive loss of its cholinesterase inhibitory (as well as alkylating) activity.

The lack of correlation between anticholinesterase activity and alkylating activity is further demonstrated by a comparison of the data in Table 2 with the results of a study presented in Fig. 7. It can be seen that the rate of reaction with NBP (i.e. the "comparative chemical alkylating activity" [16]) is relatively low for TEPA

Table 3. Main equation constants

| Compound | $K_d$ (mM) | $k_2$ ($min^{-1}$) | $k_i$ ($min^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| TEM | 0·80 | 0·077 | 0·096 |
| TEPA | 0·50 | 0·052 | 0·104 |
| AB-100 | 6·5 | 0·044 | 0·0068 |

which is a rather good cholinesterase inhibitor, and the highest for EI which has no measurable cholinesterase inhibitory activity.

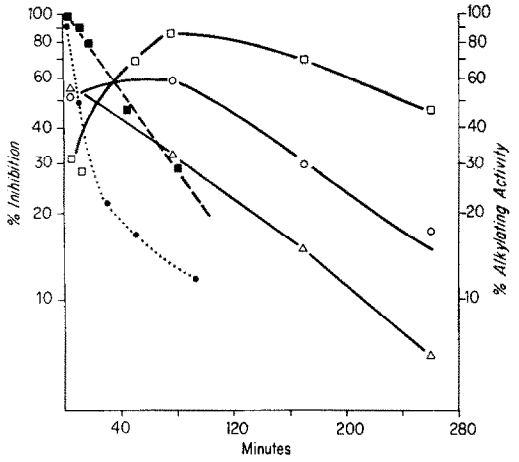



Fig. 6. Effect of prior hydrolysis of AB-163, 1·4 mM (□); AB-132, 1·5 mM (○); and TEPA-132, 1·6 mM (△) in pH 7·4, 0·066 M $NaH_2PO_4$ at 37°, for various lengths of time, on their activities as inhibitors of horse serum cholinesterase. The solid figures (■) and (●) represent the remaining 4-(*p*-nitrobenzyl)pyridine reactivity (alkylating activity) of AB-163 and AB-132, respectively, determined at various times during hydrolysis.

## DISCUSSION

The alkylating agents studied in these experiments represent at least three different types of cholinesterase inhibitors: (1) CTX is a competitive, reversible inhibitor of the enzyme; (2) ring-C-unsubstituted aziridines (TEM, TEPA and AB-100) act as irreversible inhibitors which cause progressive inactivation of the enzyme; and (3) the 2,2-dimethylaziridine derivatives also act as irreversible inhibitors, but their rapid hydrolysis modifies their over-all inhibitory effects. Thus, AB-132 and AB-163 form hydrolytic intermediates which have little or no alkylating activity but are potent, irreversible cholinesterase inhibitors, while TEPA-132 appears to form only inactive products upon hydrolysis. Therefore, the data must be interpreted in view of these different mechanisms.

The observation that CTX is a reversible inhibitor (i.e. that it does not form covalent bonds) is consistent with the well-established fact that it has no chemical alkylating activity and is, indeed, void of cytostatic activity *per se* but requires metabolic conversion to alkylating substances in order to exert its chemotherapeutic effects [18–21]. However, the relatively high potency of CTX as a competitive inhibitor of cholinesterase is difficult to explain. It shares some structural properties with butyrylcholine, but it is a much more potent inhibitor than would be expected on the basis of this structural analogy. Since CTX, as such, does not form an aziridinium ion, and its much too weakly basic phosphoramide-nitrogens would not be expected to provide for significant coulombic interaction with the anionic site of the enzyme, we tenta-

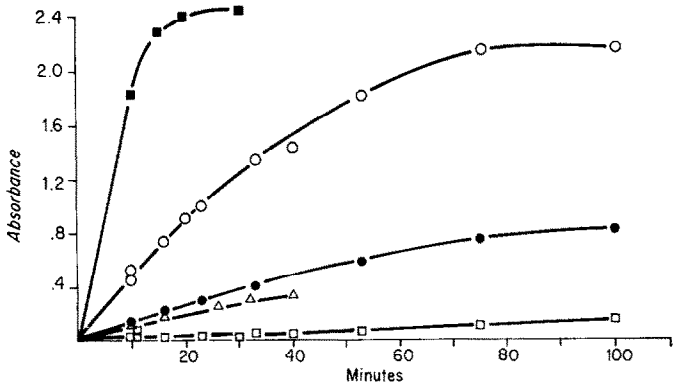



Fig. 7. Rate of alkylation of NBP by EI (■), TEM (○), AB-132 (●), AB-100 (△), and TEPA (□). The solutions of all alkylating agents contained 0·45 m-equiv of alkylating functional groups/liter (see Methods).

tively attribute the low $K_i$ value of this inhibitor in part to the hydrophobic binding ability of its unionized $\beta$-chloroethyl side chains.

It is interesting that all the aziridine type alkylating agents caused irreversible inactivation of the enzyme, and yet their inhibitory activities did not correlate well with their chemical alkylating activities as measured with NBP. It appears that the formation of a reversible complex between the alkylating agent and the enzyme is a significant factor in the anticholinesterase activities of at least some of these compounds, as it has been found previously in the case of the organophosphates and carbamates [13, 14]. This is further indicated by the applicability of the Main equation to describe the inhibitory effects of the more stable (C-unsubstituted) aziridine derivatives, and by the fact that the significantly higher anticholinesterase activities of TEPA and TEM as compared to AB-100 appear to be related to the difference in their dissociation constants ($K_d$, see Table 3). It should be pointed out that these agents have $k_2$ values several orders of magnitude lower than the typical organophosphate inhibitors. This is consistent with the much lower reactivity of the serine hydroxyl group at the catalytic site of cholinesterase toward alkylation than toward phosphorylation; however, irreversible binding of the alkylating agents might occur at some other, more nucleophilic site of the enzyme and cause conformational changes affecting the catalytic site [22]. In this case, the resulting inhibition of enzymic activity would depend in a decisive manner on specific structural (stereochemical) properties of the alkylating agent as well as of the enzyme and substrate used in the assay. Certain aziridine and $\beta$-chloroethylamine derivatives of specific structures have been used previously in studies of the binding sites of cholinesterases [22, 23].

The 2,2-dimethylaziridine-containing alkylating agents show progressive inhibition of cholinesterase with relatively low $i_{50}$ values (see Table 2), but their activities cannot be directly compared to those of their unsubstituted analogs because: (1) their very fast hydrolysis significantly decreases the values obtained for longer contact time; and (2) in the case of at least two of these agents, there is evidence (Fig. 6) indicating the formation of an intermediate hydrolysis product(s) having very high anticholinesterase activity. The complexity of the interaction of these two factors makes the kinetic analysis of the data for these agents extremely difficult. The transient appearance of a maximum of cholinesterase inhibitory activity, with continuously decreasing alkylating reactivity, during the hydrolysis of AB-163 as well as AB-132, supports our hypothesis of the formation of 5-membered cyclic intermediates similar to HX (Table 1) which, assuming analogous mechanisms of hydrolysis, could be a hydrolytic intermediate common to both drugs.* On the basis of its postulated structure, HX would not be expected to have any alkylating activity, but it could inhibit the enzyme via phosphorylation. However, such compounds are labile in aqueous solution, and they may even equilibrate between various forms (e.g. via pseudorotation) before complete hydrolysis (to HP); at any rate, all efforts to isolate HX in pure form have been unsuccessful to date.

Finally, it should be noted, that due to the relatively low hemopoietic toxicity of AB-132, this drug has been employed at considerably higher dose levels in the treatment of cancer patients than any other alkylating agents. This may be another reason, in addition to its above discussed hydrolytic intermediate, why in the case of AB-132 the anticholinesterase activity of this drug was so prominently demonstrated during the clinical trials, as a major mechanism or its dose-limiting side effects. In the case of CTX, the usual clinical dose levels are also relatively high in comparison to those of the directly acting alkylating agents; in addition, CTX is known to be converted *in vivo* to a metabolite(s) having alkylating [18–21] (and, possibly also phosphorylating [10] activities, which could inhibit the cholinesterases in an irreversible manner. In view of the rapid metabolism of CTX, the clinically observed cholinesterase inhibitory effects [7–9] of this drug are probably due to the action of its metabolites rather than to the potent but kinetically reversible inhibitory action of CTX itself which we described in the present paper.

* It was found recently that HP (see Table 1) is a final hydrolysis product of both AB-163 and AB-132, but not of TEPA-132; in the latter, all P—N bonds remain intact during the ring-opening hydrolysis of the aziridine moieties (Z. F. Chmielewicz and T. J. Bardos, unpublished observations).

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