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EFFECTS OF ALKYLATING AGENTS ON HUMAN PLASMA CHOLINESTERASE

THE ROLE OF SULFHYDRYL GROUPS IN ITS ACTIVE CENTER

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Abstract—Several alkylating agents (bromobenzylcyanide, chloroacetophenon, and xylyl bromide) were found to inhibit cholinesterase from human plasma. Less reactive alkylating compounds such as bromoacetone, chloroacetate, and iodoacetate did not inhibit the enzyme. Chloropicrin, which is a powerful oxidizing agent, was not found to inhibit either. Several mercaptide-forming agents, like *p*-chloromercuribenzoic acid, diphenylaminechloroarsine, and ethyl-dichloroarsine, were effective inhibitors. *w*-Chloroacetophenon was found to be an instantaneous and noncompetitive inhibitor. The inhibition was reversible by dilution or dialysis. The degree of inhibition was higher in an aqueous medium than when a less polar solvent was used.

These findings suggest that a sulfhydryl group is not involved during the inhibition of cholinesterase by alkylating agents. The possibility of a reaction on the histidine or methionine groups of the enzyme is discussed. The inhibition also was dependent on the substrate employed during the activity assay. The order of percentage of inhibition was found to be acetylcholine > butyrylcholine > benzoylcholine. These data are used to offer an interpretation for some aspects of the lachrymatory action of these agents.

DURING a previous investigation, it was demonstrated that *w*-chloroacetophenon acts as a sulphydryl agent on several enzymes, most of which are involved in glycolysis and the tricarboxylic acid cycle. It also was observed that some alkylating agents were as strong inhibitors of cholinesterase as *p*-chloromercuribenzoic acid and arsenicals.[1] These results are not in agreement with the expected behaviour of these compounds, if the reaction is assumed to involve thiol groups.[2–4]

Other workers[5] have shown that cholinesterase is inhibited by several strong mercaptide-forming agents, but only when these agents are employed in relatively high concentration. This enzyme was also sensitive to copper ions and iodine, but oxidizing agents like *o*-iodosobenzoic acid, oxygen, or u.v. radiation do not inhibit the enzyme.[5–7] Goldstein and Doherty[8] reported that mercuric chloride inhibits the enzyme, and they assumed that the inhibition was due to an interaction with a thiol group not belonging to the active center of the enzyme.

All these conflicting observations led to an investigation of the action of *w*-chloroacetophenon and other compounds on human plasma cholinesterase. The experiments reported here were performed to elucidate the mechanism by which this agent acts. Emphasis was placed on the degree of reversibility of the inhibitory reaction.

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MATERIALS AND METHODS

Human plasma cholinesterase (ChE) was purchased from Sigma Chemical Co. and its sp. act. was 100 units per mg (1 unit hydrolyzes 10·1 μg acetylcholine/min at pH 7·4 and 37°). Acetylcholine bromide (ACh) and benzoylcholine chloride (BzCh) were purchased from L. Light Co. Butyrylcholine *p*-toluene-sulfonate (BuCh) was obtained from Nutritional Biochemicals Co. and chloromercuribenzoic acid from Sigma Chemical Co.

Bromoacetone, bromobenzylcyanide, chloropicrin, diphenylaminechloroarsine ethyldichloroarsine, and xylyl bromide were provided in neutral glass-sealed vials by H. Stolzenberg (Germany). Ethylene-glycol-dimethyl-ether (EGDE) was obtained through Ansul Chemical Co. Chloroacetophenon (CAP) (recrystallized from ethanol–water, m.p. 59) and ethyl bromoacetate (b.p. 159) were prepared as described by Sartori.[9]

Cholinesterase activity was measured colorimetrically[10] in a few early experiments but usually by automatic titration with a Radiometer SBR2/SBU1 titrigraph, according to the procedure of Jorgensen,[11] except that distilled water was used instead of saline. Unless otherwise stated, the typical assay mixture contained ACh in a concentration of 10 mM and no EGDE. All experiments were conducted at a pH of 7·2 and 25°.

In order to correct for nonenzymatic hydrolysis of the choline esters and various inhibitors, determinations were made under the same conditions used for a typical assay, except that the ChE was omitted. When EGDE was used to dissolve an inhibitor, an appropriate control experiment was conducted with the enzyme in the presence of an amount of EGDE equal to that used during the inhibition experiment.

The concentration of CAP in a saturated aqueous solution was determined by spectrophotometric measurements at 250 mμ.

RESULTS

Effect of substrate mixtures on ChE activity

The action of different substrates on the purified ChE activity was studied by using either ACh (10 mM) or BzCh (10 mM) or a mixture of both. The activities expressed in micromoles of substrate hydrolysed per minute were: for ACh, 0·345; for BzCh, 0·062, and with both substrates together, 0·100.

Effect of several compounds on ChE activity

The effects of CAP and other well documented thiol reagents on ChE were studied. In Table 1, which lists the compounds examined, the results are expressed in terms of the degree of inhibition observed. As one can see, inhibition was observed only with those alkylating agents known to be highly reactive, i.e. CAP, xylyl bromide, and bromobenzylcyanide, and also with potent mercaptide-forming agents like diphenylaminechloroarsine and chloromercuribenzoic acid.

Rate of inhibitory reaction

In order to determine the rate of inhibitory action of CAP, two different procedures were employed. In one procedure ChE was incubated with CAP for 5, 10, or 20 min (CAP concentration 4·6 mM; EGDE 10%). The reaction was stopped with cysteine

(final concentration 25 mM) and the remaining enzyme was then assayed for activity by the colorimetric procedure. The ChE activity was almost equal in the three time periods studied. The results for the 5-, 10-, and 20-min experiments were 31, 28·8, and 29·8 per cent inhibition respectively.

TABLE 1. INHIBITION OF CHE BY SEVERAL COMPOUNDS

Compounds	Conc. (mM)	Inhibition (%)	Incubation time* (min)
Chloroacetophenon	1·25	50	5
p-Chloromercuribenzoate	1·30	50	5
Diphenylaminechloroarsine	0·20	50	5
Ethyldichloroarsine	1·30	27·4	5
Bromoacetone	8·68	0	30†
Bromobenzylcyanide	5·30	50	30†
Xylyl bromide	2·02	50	30†
Chloropicrin	8·30	0	30†
Iodoacetate, sodium salt	90·0	0	60
Chloroacetate, sodium salt	8·50	0	120

* Time of interaction between enzyme and inhibitor before adding substrate.
† Activity was determined by the colorimetric procedure.

To test the effects of shorter periods of incubation and the effect of simultaneous addition of substrate and inhibitor, the titrigraphic procedure was employed under the following conditions: ACh, 88 mM; CAP, 2·2 mM; and EGDE 5%.

Even in the presence of the substrate, the inhibition was almost immediate, and furthermore, the slope of the activity remained constant for at least 55 min. A similar type of inhibition was observed when a totally aqueous media was used instead of the EGDE solution. When *p*-chloromercuribenzoic acid or diphenylaminechloroarsine was employed as inhibitors, it could be seen that inhibition was again immediate and that the slope of activity remained constant for a considerable time.

Relation between CAP concentration and degree of inhibition

A great difference in the degree of inhibition by CAP was observed when the results obtained in a 10% EGDE solution were compared with those of a totally aqueous media. When EGDE solutions were used, it was difficult to obtain reproducible results. Occasionally, high concentrations of the drug produced nearly 100 per cent inhibition of enzyme activity. In a totally aqueous medium, the percentage of inhibition and the velocity fell to a definite limit (Figs. 1 and 2). On the other hand, the degree of inhibition did not reach a limit if EGDE-containing solutions were employed.

Effect of substrate concentration on inhibition

Fig. 3 shows a double reciprocal plot of reaction velocity vs. substrate concentration obtained in the presence and absence of inhibitor in a totally aqueous medium (CAP concentration, 4·76 mM). The inhibition was found to be noncompetitive, type IIb

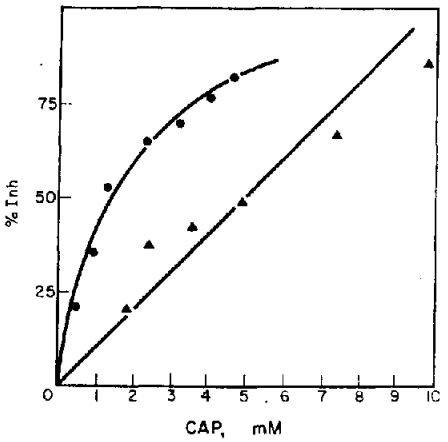

FIG. 1. Inhibition of ChE activity by various concentrations of CAP. CAP acted for 5 min on ChE, then ACh was added and activity recorded. ACh concentration 10 mM, pH 7·2 at 25°. All aqueous media = ●; media containing 10% EGDE = ▲.

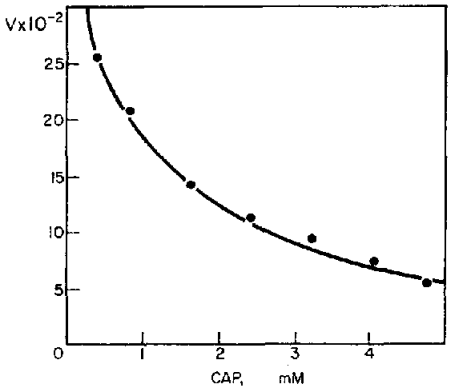

FIG. 2. Velocity of enzymatic hydrolysis of ACh in presence of various concentrations of CAP. CAP acted for 5 min on ChE, then ACh was added and activity recorded. ACh concentration 10 mM, pH 7·2 at 25°. All aqueous media = ●.

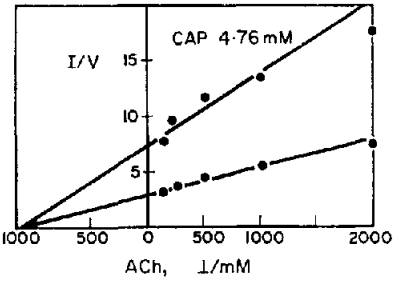

FIG. 3. Lineweaver-Burk plot for the inhibition of ChE by CAP. After 5-min incubation of CAP with ChE, ACh was added to reach the appropriate concentration, and its activity was recorded. All determinations were made at pH 7·2, 25°, and CAP concentration of 4·76 mM. All aqueous media = ●.

according to Dixon and Webb.[12] The K_m value was found to be 0·91 mM. To obtain K_i the following equation was employed:

$$K_i = \frac{i}{\left(\frac{V}{Vp} - 1\right)} \left(1 - \frac{V'}{Vp}\right)$$

V and Vp were obtained from Fig. 3, and V' was obtained by extrapolation of results shown in Fig. 2 to infinite inhibitor concentration. The K_i value was found to be 1·04 mM.

In another set of experiments, whether CAP (0·48 mM) was incubated with ChE in the absence or presence of ACh (10 mM), similar percentages of inhibition were found. For example, in the presence of ACh, 28·3 per cent inhibition was observed whereas without ACh, 29·3 per cent inhibition was noted. These results strongly support the idea that the inhibition is noncompetitive.

Effect of different substrates on degree of inhibition

The degree of inhibition was found to be dependent on the substrate employed. In one set of experiments CAP (4·76 mM) was incubated with ChE for 5 min, the substrate was added to make a final concentration of 10 mM, and the activity was recorded. The results in terms of per cent inhibition were for ACh, 76·9 and for BuCh, 54·1. When BzCh was used as a substrate, there was no inhibition, and in fact BzCh appeared to produce a 31 per cent enhancement of enzyme activity. Similar results were obtained when substrate and inhibitor were added simultaneously to the enzyme mixture. The relative inhibition potencies were the same despite the different experimental conditions (ACh > BuCh > BzCh). In a medium containing 10% EGDE (CAP 2·7 mM) the following results, expressed as per cent inhibition, were obtained: for ACh, 62·2; for BuCh, 50·8; and with BzCh, no inhibition was observed.

When a mercaptide-forming inhibitor such as diphenylaminechloroarsine (concentration, 0·5 mM) was preincubated with ChE for 5 min before substrate addition, the same order of inhibition was observed: with ACh, 78·2 per cent; with BuCh, 51·5 per cent and with BzCh, 37·4 per cent. In the latter case, inhibition was observed with BzCh.

Effect of dialysis on inhibition

If the reaction mixture is subjected to dialysis in the cold for 24 hr before adding ACh, no inhibition is observed when comparison is made against an activity assay made under the same experimental conditions. When ACh was present during the inhibition (EGDE, 5%) in an experiment similar to the previous one, a notable effect of dialysis was observed. Before dialysis, there was 54·6 per cent inhibition of ChE activity, whereas after dialysis there was no inhibition. If a totally aqueous medium (CAP 4·7 mM) was used, there was 76·9 per cent inhibition before dialysis and 0 per cent inhibition after dialysis.

Effect of dilution on inhibition

A noticeable amount of reactivation in cholinesterase activity was observed when the reaction mixture was diluted several-fold with water after an inhibition measurement. For example, before dilution 60·6 per cent inhibition was obtained (CAP, 2·2 mM; ACh, 88 mM) and after 4-fold dilution the inhibition fell to 31·6 per cent.

DISCUSSION

Role of the sulfhydryl groups in the inhibition of ChE by CAP

From the data reported in the present communication, it appears probable that the inhibition of ChE by CAP does not involve SH groups for the four reasons listed below. (1) Mercury compounds are usually more potent SH inhibitors than either the alkylating agents or the arsenicals,[2–4] and the results in Table 1 show that this is not the case, because *p*-chloromercuribenzoic acid was a less potent inhibitor than CAP or diphenylaminechloroarsine. (2) A powerful oxidizing agent like chloropicrin does not inhibit as would be expected for a reaction involving SH groups. In accordance with this result, other authors[5,6] have reported that another oxidizing agent, O-iodosobenzoic acid does not inhibit the ChE. (3) The inhibition by arsenicals is not due to interaction on vicinal SH groups as could be supposed. This possibility could be ruled out by comparison of the relative potencies of the arsenicals to oxidants, since the vicinal SH groups might well react with both types of inhibitors. Usually oxidants are about five times more potent than the arsenicals.[4] This was not the case in this study, since ChE was found to be highly sensitive to arsenicals whereas chloropicrin, which is a powerful oxidizing agent, did not inhibit the enzyme. (4) The reaction of ChE with chloroacetophenon was shown to be reversible by the following experimental results. (a) When the hydrolysis of ACh in the presence of ChE and CAP was followed continuously, no changes in rate were observed over a period of 55 min, suggesting that no increase in inhibition occurred. (b) The ChE activity that was inhibited by CAP could be totally restored by dialysis. (c) The degree of inhibition by CAP can be considerably reversed by dilution.

It is therefore concluded that the reaction must be reversible since, if an irreversible product were formed with ChE, the inhibition would be expected to increase with time and should persist after dilution and dialysis. These findings give a clear indication that the action of CAP on ChE does not involve SH groups because the S–C covalent bond produced in this type of reaction can be broken only by heat or by powerful chemicals. Such a bond can not be ruptured by mild reagents at room temperature or simply by dilution or dialysis.

Nature of the reacting group during inhibition of ChE by CAP

Besides the action on SH groups, alkylating agents can be expected to undergo other reactions with proteins such as: (1) ε-amino groups of lysine residues, (2) imidazol groups of histidine residues, (3) the phenolic hydroxyls of tyrosine, or (4) with thio-ether groups of methionine.[13,14] These four reactions are generally slower than that occurring with sulfhydryl groups, particularly at neutral or slightly acid pH.[14]

It is therefore important to note that, during similar studies on chymotrypsin, Schramm and Lawson[15] reported that an alkylation occurred between *w*-bromoacetophenon and methionine groups of the enzyme and that it is well established that other alkylating agents like *bis*-(β-chloroethylsulfide) react with methionine to form a labile sulfonium salt which is reversibly decomposed.[16] But alkylation of methionine more likely occurs at a pH level below 5·5 than at neutral or slightly acidic pH values. The most rapid reaction under the two latter conditions should be with the imidazol group of the histidine residue, because the imidazol groups are totally in the active form to react with alylating agents at this pH. It is significant that other workers[17]

have observed that compounds quite similar to *w*-chloroacetophenon, for example the chloromethyl derivative of *n*-tosyl-l-phenylalanin, inhibit chymotrypsin by alkylation of a histidine moiety essential for the enzyme action. A similar action of this compound was proposed to occur with trypsin.[18] Reversibility by dilution and dialysis is easier to explain if a reaction with imidazol or thio-ether groups is supposed.

The effect of EGDE on inhibition did not provide a method by which one could discriminate between a reaction on histidine or methionine, because the inhibitory reaction seems to be unimolecular. This can be deduced from the rapid inhibition of ChE by CAP and the lack of dependence of the percentage of inhibition on enzyme concentration. (A 5-fold increase in enzyme concentration did not significantly modify the percentage of inhibition.)

In a unimolecular reaction, the rate of reaction depends only on the breaking of the covalent bond and therefore the increase in solvent polarity would produce an increase in the overall rate. Because there are no differences in the solvent effect on the rate of alkylation of methionine and histidine, this test can not be applied to determine which of these amino acids is involved in the inhibitory reaction.

In summary, it can be stated that the reaction of CAP on ChE does not involve SH groups; but from these and other studies, it may be suggested that other groups like the thio-ether residue from methionine and the imidazol group of histidine might be involved in the interaction of CAP on ChE.

Site of the reacting group

Two lines of evidence suggest that the chemical reaction leading to inhibition of the enzyme activity involves a chemical group not belonging to the active center of the enzyme. First, the data from the Lineweaver–Burk plot (Fig. 3) offer clear evidence for a mechanism of noncompetitive inhibition. Second, it was found that the percentage of inhibition by CAP was essentially the same in the presence or absence of substrate during inhibition. It is concluded from these experiments that the group being alkylated does not belong to the active center of the enzyme.

Mechanism of inhibition of ChE by CAP

In considering the possible mechanism of inhibition, it may be seen from Fig. 2 that when the CAP concentration is increased in an aqueous medium, the velocity falls to a definite limit, but not to zero. According to Dixon and Webb,[19] this behaviour corresponds to the non–competitive inhibition designated as type IIb. In this type of inhibition the enzyme–substrate–inhibitor complex (EIS) may break down at a different velocity from the enzyme–substrate complex (ES), and the velocity is the sum of the two reactions. The velocity is given by the following expression:

$$v = \frac{k.e\left(1 + \frac{i}{K_i}.\frac{k'}{k}\right)}{\left(1 + \frac{K_s}{s}\right)\left(1 + \frac{i}{K_i}\right)}$$

where k is the rate constant for the decomposition of (ES), and k' is the rate constant for the decomposition of EIS. If k' is zero, this equation reduces to the usual non-competitive one, but if k' is greater than k, the inhibitor can act as an activator of the enzyme. The effect of the different substrates used in this work can be explained in

terms of changes in k'. This means that the rate constant k' should be greater than or equal to k for benzoylcholine, and smaller than k for butyrylcholine and acetylcholine.

Alternatively, it may be suggested that steric factors are also important, since the sequence in percentage of inhibition was BzCh < BuCh < ACh and the sequence of the molecular volumes for the same substrates is BzCh > BuCh > ACh. These results suggest that a certain degree of protection is afforded to the enzyme by steric factors. Substrate affinity does not seem to play an important role, because the affinity sequence for the plasma enzyme is BuCh > ACh > BzCh,[20] and the sequence in percentage of inhibition is ACh > BuCh > BzCh.

Some of the results obtained in this study could be interpreted as due to the presence of more than one enzyme. However, the experiments in which the behaviour of ChE was tested in the presence of substrate mixtures, as was recommended by Augustinsson,[20] showed a strong competitive effect due to interaction of both substrates on the same enzyme. Therefore, it can be concluded that in the preparation used in this work one enzyme predominates and the results are attributable to this enzyme.

Possible relation with the biochemical lesion produced by lachrymators

Dixon postulated that lachrymation is primarily due to a rapid, progressive and irreversible attack on SH groups of the cornea.[21] This was assumed to be due to the fact that the S–C covalent bond formed during the reaction of the lachrymators with the SH groups is quite stable. However, as Peters[22] pointed out, it is quite difficult to reconcile this idea with the fact that lachrymatory action wears off by tears when exposure ceases. In this work, a rapid and reversible reaction between a typical lachrymator like CAP and a chemical group of ChE was found. If a reaction with a similar chemical group of the cornea occurs during lachrymation, the objections of the hypotheses of Dixon would be overcome. During his studies, Dixon[21] found that lachrymators give raise to trains of electrical impulses in the corneal nerve fibres and he offered no explanation for this fact.[8] Inhibition of cholinesterase in the cornea could explain that observation. In behalf of this possibility, a high choline acetylase and cholinesterase content was found recently in the epithelium of bovine cornea.[23]

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