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EFFECT OF HISTIDINE MODIFICATION ON THE AGING OF ORGANOPHOSPHATE-INHIBITED ACETYLCHOLINESTERASE

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Abstract—Organophosphate-inhibited bovine erythrocyte acetylcholinesterase undergoes aging, a process whereby the phosphoenzyme is transformed to a form which is no longer reactivated by oximes. Aging was estimated indirectly by reactivation of trichlorfon-inhibited acetylcholinesterase by pyridine-2-aldoxime methiodide or directly by loss of tritium from [1,3-^{3}H]diisopropylfluorophosphate inhibited enzyme. The aging of diisopropylfluorophosphate(DFP)-inhibited acetylcholinesterase was due primarily to dealkylation, rather than to the alternative β-elimination mechanism which was also examined. Aging of trichlorfon-inhibited acetylcholinesterase was not decreased by modification of sulfhydryl, carboxyl or amino groups outside the active site of the enzyme. Modification of histidine by 2 mM diethylpyrocarbonate irreversibly inhibited acetylcholinesterase activity. Dealkylation of [1,3-^{3}H]DFP-inhibited acetylcholinesterase was slowed 2-to 3-fold by reaction of active site histidine with 2 mM diethylpyrocarbonate, and it was abolished at higher concentrations. It was concluded that an active site histidine catalyzes both aging and acetylcholine hydrolysis in erythrocyte acetylcholinesterase.

Organophosphates, used widely as insecticides and to a lesser extent as therapeutic agents, inactivate acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by phosphorylation of a serine hydroxyl at the active site. Nucleophiles, such as 2-PAM,† reverse the inhibition of certain organophosphates and may be effective antidotes of organophosphate poisoning *in vivo* [1] and *in vitro* [2]. Organophosphate-inhibited acetylcholinesterase, however, undergoes a gradual irreversible transformation, known as aging, to a form that is refractory to reactivation by oximes [3]. It would be useful to determine the mechanism of aging so that approaches to the treatment of organophosphate poisoning can be developed. Although previous studies have shown that organic cations [4] and organic solvents [5] retard the rate of enzyme aging, the therapeutic usefulness of even the more effective agents, such as *d*-tubocurarine, alcuronium and gallamine [4], is limited by their non-specific cholinergic actions. Reported beneficial effects of such agents *in vivo* appear unrelated to their acetylcholinesterase actions [6].

Aging of acetylcholinesterase follows first-order kinetics [7] and has generally been associated with dealkylation [8, 9] by C—O [10, 11] or P—O [12, 13] fission of a phosphate substituent. Dealkylation appears to be an enzyme-catalyzed reaction, as it requires an active enzyme conformation [2, 11, 14] and is modulated by certain allosteric effectors of the enzyme [4, 5]. Aging of methylphosphorylated-acetylcholinesterase is catalyzed by a group on the enzyme of pK_a 5.8 [15] or 6.4 [11], similar to the pK_a of around 6 found for catalysis of acetylcholine hydrolysis [16, 17]. The involvement of a carboxyl group in aging has been inferred from thermodynamic data [15] but has not been shown directly. In the present study the effects of a number of amino acid modifying reagents on the aging of organophosphate-inhibited bovine erythrocyte acetylcholinesterase have been investigated. It is shown that modification of histidine in the active site inhibits dealkylation.

MATERIALS AND METHODS

Purification of acetylcholinesterase. Bovine erythrocyte acetylcholinesterase [Sigma Type 1, sp. act. 3.3 μmoles acetylcholine hydrolyzed/min (3.3 units) per mg solid] was dissolved in 0.1 M NaCl and 20 mM sodium phosphate, pH 7.4, at a concentration of 1 mg/ml. A sample (1000 units) was applied to a 10-ml affinity column of phenyltrimethylammonium liganded to Sepharose 2B through the meta-position [18], at a rate of 0.5 column vol./hr as described previously [19]. The specific activity of the enzyme was 30–60 units/mg protein.

2-PAM reactivation. Solid trichlorfon was added, in a final concentration of 0.8 mM, to 2 ml of Sigma bovine erythrocyte acetylcholinesterase (6.6 units/ml) in 1 mM sodium phosphate buffer, pH 7.4, at 25°. After phosphorylation of the enzyme was essen-

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† Abbreviations: 2-PAM, pyridine 2-aldoxime methiodide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DEPC, diethylpyrocarbonate (ethoxyformic anhydride); DFP, diisopropylfluorophosphate; and trichlorfon (Dipterex), dimethyl(2,2,2-trichloro-1-hydroxyethyl)-phosphate; and TCA, trichloroacetic acid.

tially complete, 125-μl aliquots were transferred at hourly intervals to 40 ml of 0.0375 mM 2-PAM in 1 mM sodium phosphate (pH 7.4) at 25°. At various time intervals (from 4 to 40 min), 100-μl aliquots of the 2-PAM-treated enzyme were transferred to 100 μl of a mixture of [acetyl-^{14}C]choline iodide (38 nCi) and 1 mM acetylcholine iodide in 0.2 M sodium phosphate buffer, pH 7.4. The reaction was terminated with 200 μl ethanol after 20 min and the remaining enzyme activity was determined as described previously [20]. The maximum activity recovered after 2-PAM treatment was determined from the intercept on the ordinate of plots of 1/activity vs 1/time. The log of the maximum activity (log $V'_{(max)}$) after 2-PAM treatment was plotted against time before 2-PAM reactivation. The ordinate intercept from the line of best fit yielded the best estimate of the maximum reactivation by 2-PAM at zero time (no aging) ($V'_{o(max)}$). A plot of log ($V'_{(max)}/V'_{0(max)}$) vs time before 2-PAM reactivation yields the rate and half-life ($T_{\frac{1}{2}}$) of aging (see Fig. 1).

Identification of the products of aging. Purified acetylcholinesterase (0.7 units/ml) in 1.0 ml of 0.1 M sodium phosphate (pH 7.4) was reacted with 2.5 μl of [1,3-^{3}H]DFP (10 mM) for 10 min at 29°, followed in some instances by reaction with 10 μl DEPC (2 mM final concentration, freshly prepared in 10% ethanol) for a further 10 min at 29°. The mixture was applied to a Sephadex G-10 column (2 × 22 cm) and eluted with 0.1 M sodium phosphate, pH 7.4. Fractions (twenty drops) were collected and 100-μl samples were counted in a scintillation counter. Three fractions with the highest counts (fractions 22–24) from the first elution peak were pooled and incubated at 29° for 7 hr. A sample (1.5 ml) of this mixture was applied to the Sephadex G-10 column (previously washed to remove most of the residual [1,3-^{3}H]DFP) and eluted with 0.1 M sodium phosphate, pH 7.4. Aliquots (0.6 ml) from each fraction were counted in 10 ml ACS scintillation fluid.

Measurement of dealkylation rate. Acetylcholinesterase (200 μl, 7 units/ml) was diluted with 300 μl of 0.1 M sodium phosphate buffer (pH 7.4) and reacted with [1,3-^{3}H]DFP at a final concentration of 10 mM for 10 min at 29° and then with 2–10 mM DEPC for a further 10 min at 29°. Unreacted [1,3-^{3}H]DFP and DEPC were separated from the phosphorylated enzyme on a Sephadex G-50 column (0.6 × 29 cm) eluted in a descending manner with 0.1 M sodium phosphate (pH 7.4) at a pressure of 6 cm of water. The fractions containing [1,3-^{3}H]DFP-reacted acetylcholinesterase were pooled (1.5 to 2.0 ml) and incubated at 29° in the same buffer. The enzyme activity in these fractions was found to be 99 per cent inhibited by the DFP treatment. At various times, 200-μl samples were removed and added to 1 ml of 6% TCA and filtered by suction through a 0.22-μm Millipore filter (type GS). The protein was washed three times with 1 ml 5% TCA. The filter was dried at 90° for 15 min, solubilized with 10 ml Aquasol scintillation fluid, and counted for tritium.

Acetylcholinesterase assay. Acetylcholinesterase (20 μl) was assayed as described previously [20, 21], by incubation for 10 min at 29° in 180 μl sodium phosphate (pH 7.4) and 20 μl of a mixture of acetylcholine iodide and [acetyl-1-^{14}C]choline iodide, at a final concentration of 1.0 mM acetylcholine.

Source of chemicals. Trichlorfon was supplied by the Defence Research Board of Canada (Suffield, Alberta). Bovine erythrocyte acetylcholinesterase, diethylpyrocarbonate, EDAC and acetylcholine iodide were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. 2-PAM was from Koch-Light Laboratories, Colnbrook, U.K., and acetyl-[1-^{14}C]choline iodide (1–5 Ci/mole) from the New England Nuclear Corp., Lachine, Quebec, Canada. [1,3-^{3}H]DFP (3.3 Ci/mmole) and [U-^{14}C]isopropanol (0.49 Ci/mole) were from the Radiochemical Centre, Amersham, U.K. Iodoacetamide was from the Nutritional Biochemical Co., Cleveland, OH, U.S.A. Sephadex G-10 and G-50 were obtained from Pharmacia, Uppsala, Sweden.

RESULTS

Aging of phosphorylated acetylcholinesterase was studied initially by measurement of 2-PAM reactivation of trichlorfon-inhibited enzyme. As DEPC modification irreversibly inhibited acetylcholinesterase activity, aging was also studied directly as loss of radioactivity from [1,3-^{3}H]DFP-labeled enzyme.

2-PAM reactivation. The aging of acetylcholinesterase inhibited by trichlorfon was assessed by estimating the rate of loss of reactivation of the phosphorylated enzyme by 2-PAM, as shown in Fig. 1 for the control enzyme ($T_{\frac{1}{2}}$ = 12.8 hr) and for enzyme modified by 2 mM EDAC ($T_{\frac{1}{2}}$ = 5.7 hr). The effects of covalent modification of acetylcholinesterase on the $T_{\frac{1}{2}}$ of phosphorylation and dephosphorylation are summarized in Table 1. EDAC slowed the phosphorylation 2- to 3-fold, whereas iodoacetamide had no effect. EDAC increased the aging rate 2-fold, whereas iodoacetamide probably had little effect overall. The effect of DEPC could not be investigated by this method, since it inhibited acetylcholinesterase catalytic activity. The DEPC inhibition was probably due to modification of histidine(s) in the active site [17], as it was prevented in the presence of acetylcholine. Incubation of acetylcholinesterase with 2 mM DEPC in the presence of 10 mM acetylcholine had little effect on phosphorylation or the rate of aging, probably because the active site was protected from reaction with DEPC.

Mechanism of aging. Aging could occur by at least two mechanisms, dealkylation [9] and β-elimination [22]. Aging of [1,3-^{3}H]DFP-inhibited acetylcholinesterase will yield [1,3-^{3}H]isopropanol by the former mechanism and [1,3-^{3}H]diisopropylphosphoric acid by the latter. The products of aging were identified on a Sephadex G-10 column calibrated with [^{14}C]isopropanol and [1,3-^{3}H]diisopropylphosphoric acid (produced by alkaline hydrolysis of [1,3-^{3}H]DFP for 24 hr or more at room temperature). Following reaction of acetylcholinesterase with [1,3-^{3}H]DFP, excess reagent was separated from the enzyme on a Sephadex G-10 column. The phosphorylated enzyme fraction was incubated for 7 hr at 29° in 0.1 M sodium phosphate, pH 7.4, and reapplied to the Sephadex G-10 column. The predominant prod-

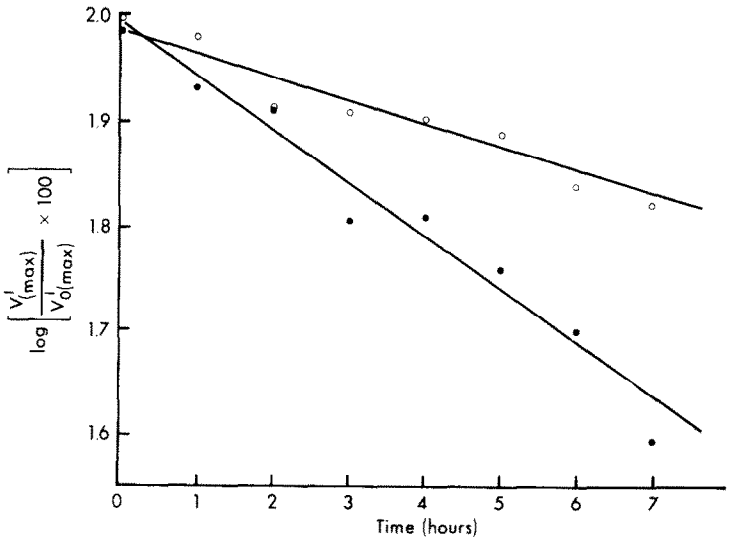

Fig. 1. Aging of trichlorfon-inhibited acetylcholinesterase. Aging was determined from the loss of 2-PAM reactivation (see Materials and Methods) for the control enzyme (○), and after modification by 2 mM EDAC (●). Lines were drawn by linear regression analysis. Single experiments typical of two similar experiments (Table 1) are shown.

uct released corresponded to the elution peak of isopropanol (Fig. 2). A smaller amount of radioactivity corresponding to diisopropylphosphoric acid varied between experiments, and it could be fully accounted for by contamination of the phosphorylated enzyme fraction with trace amounts of [1,3-^{3}H]DFP not removed by the initial Sephadex separation. In Fig. 2 the estimated contamination (from the initial elution profile of the Sephadex G-10 separation of unreacted [^{3}H]DFP) was 132 cpm, which corresponded closely to the radioactivity of the diisopropylphosphoric acid peak (140 cpm). Thus, dealkylation appeared to be the major mechanism of aging.

Effect of diethylpyrocarbonate modification on aging. Maximum inhibition of acetylcholinesterase activity (>95 per cent) occurred with DEPC after 10 min at 29° (Fig. 3A). The inhibition was irreversible, as the enzyme, separated from excess DEPC on a Sephadex G-10 column, remained inhibited after incubation in 0.1 M sodium phosphate buffer, pH 7.4, at 29° for 24 hr or more and dialysis for 16 hr against 500 ml of the same buffer (results not shown). A number of criteria suggested that DEPC reacted with the active site of the enzyme; acetylcholine (10 mM) protected against the inhibition, and the DEPC-modified enzyme incorporated only 18 per cent [1,3-^{3}H]DFP compared to the control enzyme

Table 1. Effect of acetylcholinesterase modification on the aging of trichlorfon-inhibited enzyme*

	Half-life ($T_{\frac{1}{2}}$)	
	Phosphorylation (min)	Aging (hr)
Control	4.9–9.0	10.5–12.8
EDAC	13.7–17.5	5.7–6.9
DEPC + ACh	8.9	13.7
Iodoacetamide	6.0	5.6–9.2

* Acetylcholinesterase was reacted with 2 mM EDAC for 3 hr at pH 6.0, with 2 mM DEPC in the presence of 10 mM acetylcholine (ACh) for 10 min at pH 7.4, and with 1 mM iodoacetamide for 45 min at 25° and pH 7.4 in 1 mM sodium phosphate buffer. The treated enzyme was dialyzed against 1 mM sodium phosphate (pH 7.4) at 4° for 17 hr in each case. Aging was estimated by linear regression analysis of first-order plots of the type shown in Fig. 1. The correlation coefficient of the regression lines was generally between 0.94 and 0.99. The range of duplicate experiments is given, except where single determinations were made.

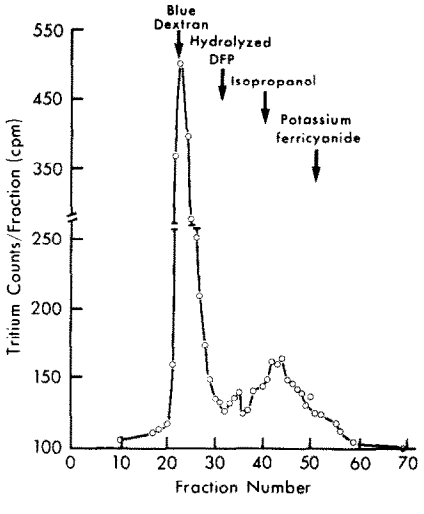

Fig. 2. Sephadex G-10 chromatography of the products of dealkylation of [1,3-^{3}H]DFP-inhibited acetylcholinesterase. Aging was allowed to occur for 7 hr at 29°. The elution peaks of the agents used to calibrate the column are indicated by the arrows, and are mean values of two to three separate experiments which varied within three fractions.

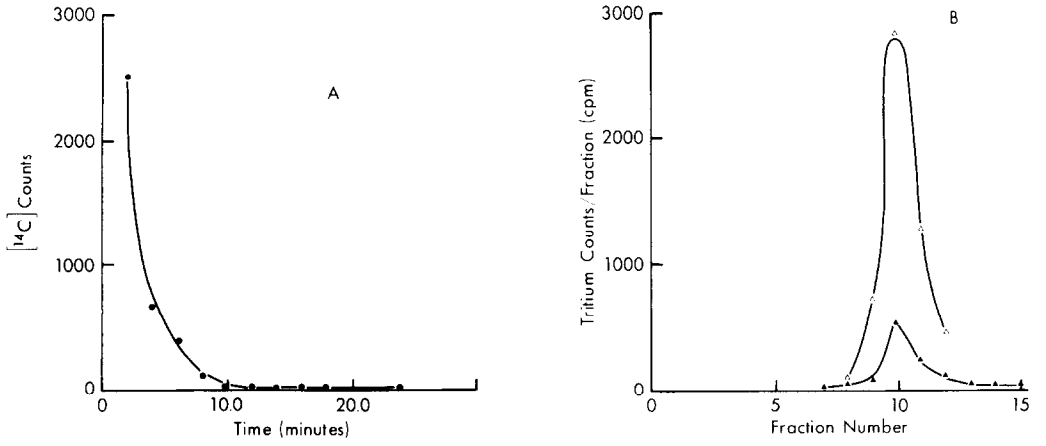

Fig. 3. Inhibition of acetylcholinesterase activity (A) and [1,3-^{3}H]DFP incorporation (B) by diethylpyrocarbonate. In Panel A, the acetylcholinesterase activity (^{14}C-counts from the hydrolysis of [acetyl-1-^{14}C]choline) was measured in the presence of 2 mM DEPC preincubated with acetylcholinesterase for various times as shown. In Panel B is shown the elution profile of the enzyme after incorporation of [1,3-^{3}H]DFP in the absence (△) and in the presence of 2 mM DEPC for 10 min (▲). Incorporation of [1,3-^{3}H]DFP was measured by the Millipore filtration procedure described in Materials and Methods.

(Fig. 3B). DEPC appeared to inhibit by carbethoxylation of a histidine-imidazole [17, 23], as reaction with 100 mM hydroxylamine for 10 min at 29° regenerated enzyme activity (results not shown).

The effect of 2 mM DEPC modification of [1,3-^{3}H]DFP-inhibited acetylcholinesterase on the rate of dealkylation is shown in Fig. 4. Dealkylation followed first-order kinetics. DEPC increased the half-life of dealkylation from 6.7 ± 0.8 hr (N = 5) to 16.5 ± 5.1 hr (N = 3). The decrease in the rate of dealkylation by 2 mM DEPC was statistically significant ($P < 0.005$) by Students *t*-test. DEPC (10 mM) almost completely abolished the dealkylation in a duplicate experiment (results not shown).

Effect of diethylpyrocarbonate modification on the product of aging. To determine if the product of aging after 2 mM DEPC was the same as that before DEPC modification, the [1,3-^{3}H]DFP-inhibited acetylcholinesterase treated with 2 mM DEPC was allowed to age for approximately one half-life (20 hr), and the products were analyzed on a Sephadex G-10 column (Fig. 5). As with the enzyme before modification, all of the radioactivity corresponded to the elution volume of isopropanol.

DISCUSSION

In agreement with previous studies [9–11], the major pathway of the decomposition of diisopropylphosphate- inhibited bovine erythrocyte acetylcholinesterase was shown to be dealkylation. It has been suggested that the catalytic group responsible for this reaction is a protonated amino acid [24] or an undissociated carboxyl group [14, 15]. Modification of peripheral carboxyl groups on acetylcholinesterase by 2 mM EDAC, under conditions that

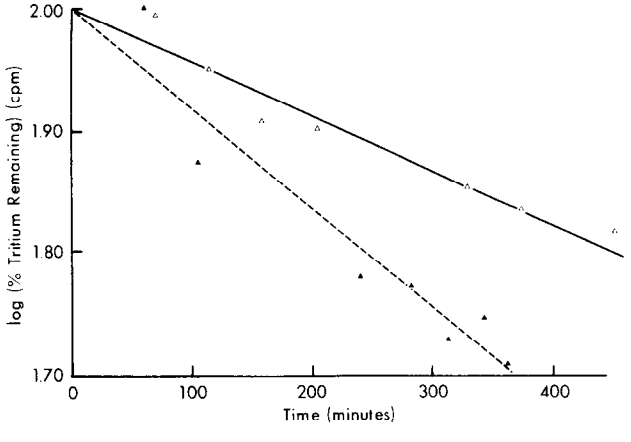

Fig. 4. Effect of diethylpyrocarbonate on the rate of dealkylation of [1,3-^{3}H]DFP-inhibited acetylcholinesterase. Key: control (▲); and 2 mM DEPC (△).

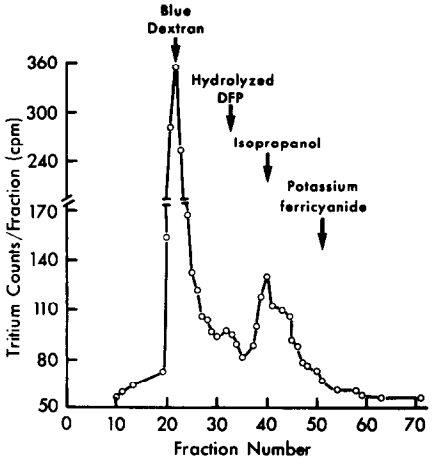

Fig. 5. Sephadex G-10 chromatography of the products of [1,3-^{3}H]DFP-inhibited acetylcholinesterase after 2 mM DEPC modification. The DEPC-modified enzyme was allowed to dealkylate for 20 hr at 29° The elution peaks of the agents used to calibrate the column are indicated by the arrows, and represent mean values of two to three separate experiments that varied within three fractions.

abolish allosteric effects of organic and inorganic cations on catalytic activity [20, 25], increased the rate of aging and decreased the rate of phosphorylation of the enzyme. Iodoacetamide, a relatively specific sulfhydryl group reagent [26], also increased the rate of aging without affecting the rate of phosphorylation or the rate of acetylcholine hydrolysis. In the presence of acetylcholine, which protected the active site from modification, DEPC did not affect the rate of aging, suggesting that imidazole (and possibly primary amino groups) outside the active center did not influence the aging rate.

As the group that catalyzed aging appeared to be located in the active site of the enzyme, the decomposition of the phosphoenzyme was determined by a direct method based on the procedure of Coult *et al.*[9]. Three major pathways of phosphoenzyme decomposition were considered—dephosphorylation, β-elimination [22], and dealkylation. The first mechanism was eliminated, as acetylcholinesterase activity was not regenerated in the time period studied. Significant amounts of the product expected from β-elimination, diisopropylphosphoric acid, were not produced during the aging period. The major decomposition product of the aging reaction peak corresponded to isopropanol, the major product expected from dealkylation [11]. The broadness of the elution peak suggested that other products expected from a carbonium ion mechanism [9, 11] were also produced. Thus, it was concluded that dealkylation was the major pathway of the aging reaction in the present experimental conditions.

Modification of acetylcholinesterase by DEPC inhibited aging. The results of DEPC modification in the present study were consistent with modification of active site histidine, as suggested previously [17]. Modification of histidine also partially inhibits the aging of phosphate and carbonate esters of α-chymotrypsin [27, 28]. Although aging was not completely blocked by DEPC at a concentration that completely blocked catalytic activity, aging was completely abolished at 10 mM DEPC. It is possible that DFP sterically hindered the approach of DEPC to the histidine site [22], requiring a higher DEPC concentration to achieve complete histidine modification. The partial inhibition was not due to reversal of the DEPC inhibition, because after 2 mM DEPC the enzyme activity remained inhibited during the time course of the experiments (results not shown), consistent with the reported half-life of carbethoxylated histidine of 55 hr [29].

Finally, it is of interest to consider the mechanism of histidine-catalyzed dealkylation. Dealkylation of the [1,3-^{3}H]DFP may occur by an electrophilic mechanism [11, 27] or a nucleophilic mechanism [12, 29], as shown in Fig. 6, A and B, respectively. Histidine could catalyze both pathways [5], through either the protonated imidazolinium or the ε2 nitrogen. It is possible that both mechanisms operate in dealkylation of DFP-inhibited acetylcholinesterase, although the pH dependence suggests that the electrophilic mechanism in A is the most likely. The present results indicate that selective modification of the histidine is unlikely to be feasible as a therapeutic approach to preventing loss of 2-PAM reactivation of the aged enzyme, as the same histidine group(s) appears to catalyze both aging and substrate hydrolysis.

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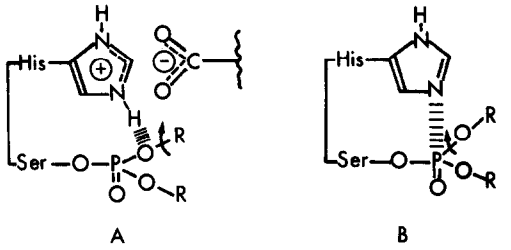

Fig. 6. Mechanisms of histidine-catalyzed dealkylation of organophosphate-inhibited acetylcholinesterase. The alkyl substituent is denoted by R, histidine by His, and serine by Ser. (A) Electrophilic substitution by an acidic group, (B) Nucleophilic substitution by the basic ε2N of histidine.

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