significantly. Triton X-100 at 0·2% was inhibitory. The enzyme activity was markedly increased by the addition of *N*-ethylmaleimide or copper. The addition of a higher concentration (0·2%) of Triton X-100 together with *N*-ethylmaleimide or copper was inhibitory, whereas Cutscum did not inhibit the activity even at 0·5%. The inhibition by Triton X-100 was more pronounced in the presence of *N*-ethylmaleimide. Dopamine β-hydroxylase activity in the washed particulate fraction was also very low. The addition of Triton X-100 (0·01%) or Cutscum (0·5%) significantly increased the activity. The addition of *N*-ethylmaleimide or copper markedly increased the activity in the washed particles. A higher concentration (0·2%) of Triton X-100 was also inhibitory, especially in the presence of *N*-ethylmaleimide. The activity in the presence of Cutscum was higher than that in the presence of Triton X-100. It was found that Triton X-100 at 0·2% inhibited the purified bovine adrenal dopamine β-hydroxylase[6] by 80%. In contrast, Cutscum did not inhibit the purified enzyme[6] even at 0·5%.

These results suggest that the endogenous inhibitors, which may be sulfhydryl compounds[1] and can be activated by *N*-ethylmaleimide or copper, exist not only in the soluble fraction, but also in the washed particles. In the presence of 0·2% Triton X-100, the activity was inhibited, and the activity was not increased by the addition of *N*-ethylmaleimide. Therefore, our present result using *N*-ethylmaleimide and Triton X-100 (0·2%) as in the experiment by Belpaire and Laduron[5] agreed with their report. However, the activity in the particulate fraction in the presence of 0·2% Triton X-100 could be increased by the addition of copper in our experiment. We have shown that by using Cutscum as a detergent, the addition of *N*-ethylmaleimide or copper greatly stimulated the activity of dopamine β-hydroxylase in the particles. This finding is consistent with our previous result[1] on the presence of endogenous inhibitors in the chromaffin granules. The possible physiological role of the endogenous inhibitors in the chromaffin granules remains to be further elucidated.

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Reactivation of isopropyl-methylphosphonylated acetylcholinesterase by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide—The effect of pH

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THE EFFICIENT reactivation by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide of bovine erythrocyte acetylcholinesterase inhibited by isopropyl-methylphosphonofluoridate was described in a previous paper.[1] This compound is 3·3 times more effective as a reactivator than trimedoxim (α,ω-bis-(4-hydroxyiminomethylpyridinium)-propane dibromide), when bovine erythrocyte acetylcholinesterase is employed as the enzyme source.[1]

To obtain more information about the reactivation mechanism we studied the influence of pH on reactivation by this oxime of bovine erythrocyte acetylcholinesterase inhibited by isopropylmethylphosphonofluoridate.

Experimental

α,ω-Bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide was prepared by a method described previously[1] and had a melting point of 255–257° (decomp.).

All other chemicals were of reagent grade.

Purified bovine erythrocyte acetylcholinesterase was used for all experiments.[2] The lyophilized preparation had a specific activity of 0·245 μmole of acetylcholine per minute per milligram at 25° and pH 8·0. The K_m of 0·58 mM for acetylcholine as substrate was estimated. Reported $K_m = 0{\cdot}60$[3] and 0·55 mM,[4] respectively.

The activity of acetylcholinesterase was determined by the electrometric method with the direct recording.[5] A 2·76-mM solution of acetylcholine iodide was used as substrate. All measurements were performed at 25°. The described apparatus[5] was used for all kinetic measurements.

The procedure used for the determination of kinetic constants of reactivation was essentially the same as that described by Wang and Braid.[6] Inhibition of acetylcholinesterase by 5 nM isopropylmethylphosphonofluoridate (30 min preincubation) was carried out at 25°. The reactivation was performed over the range of α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide concentration from 0·01 to 0·5 mM and at pH 6·60–8·26 in a 20-mM sodium phosphate buffer isotonized by sodium chloride [0·9% (w/v)] at 25°. The pH was determined with the aid of Radelkis Blood pH Meter type OP-203.

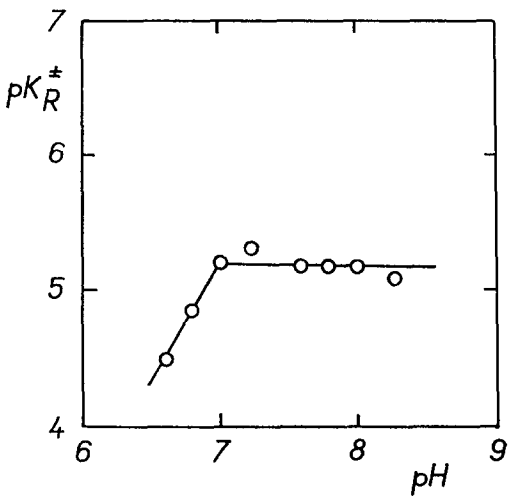

FIG. 1. Effect of pH on dissociation constant $pK_R^{\pm}$.

Results and discussion

The reaction of organophosphate inhibited acetylcholinesterase (EI) by the reactivator (R) may be represented by the scheme[7]

$$\mathrm{EI} + \mathrm{R} \underset{}{\overset{K_R}{\rightleftharpoons}} \mathrm{EIR} \xrightarrow{k_R} \mathrm{E} + \mathrm{P}.$$

In the first stage the reversible intermediate complex EIR is formed and this equilibrium is characterized by the dissociation constant K_R. In the second stage the EIR complex is broken down to the reactivated enzyme E and product P and this step is characterized by a monomolecular rate constant k_R. A bimolecular rate constant $k_r = k_R/K_R$ is a theoretical one when $[\mathrm{R}] \ll K_R$.

The kinetic constants obtained at different pH values are given in Table 1. It is seen that the decrease of values of k_R is dependent on pH. The intermediate complex EIR is more stable at higher pH.

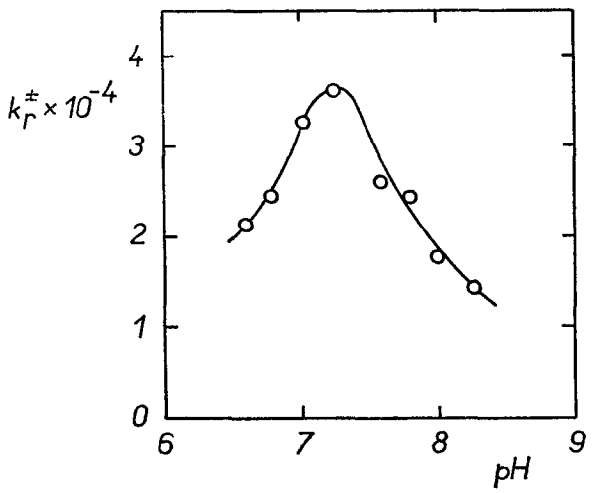

FIG. 2. Effect of pH on the bimolecular rate constant $k_r^{\pm}$. $k_r^{\pm} = k_R/K_R^{\pm}$.

TABLE 1. THE DEPENDENCE OF KINETIC CONSTANTS OF REACTIVATION OF ISOPROPYL-METHYLPHOSPHONYLATED ACETYLCHOLINESTERASE BY α,ω-BIS-(4-HYDROXYIMINOMETHYLPYRIDINIUM)-2-TRANS-BUTENE DIBROMIDE ON pH

pH	k_R (min^{-1})	K_R (μM)	k_r ($10^3 \times M^{-1} min^{-1}$)
6·60	0·668 ± 0·106	715·0 ± 121·0	0·93 ± 0·37
6·79	0·357 ± 0·038	200·0 ± 18·0	1·78 ± 0·23
7·02	0·200 ± 0·026	46·5 ± 3·9	4·40 ± 0·90
7·24	0·182 ± 0·028	27·8 ± 2·9	6·54 ± 1·86
7·59	0·178 ± 0·019	20·0 ± 1·3	8·90 ± 1·50
7·78	0·165 ± 0·021	15·0 ± 1·2	10·95 ± 1·96
7·99	0·125 ± 0·025	12·0 ± 1·1	10·20 ± 1·62
8·26	0·124 ± 0·021	11·6 ± 0·9	10·68 ± 1·12

The values are given as mean ± confidence limit for P = 0·95.

The values of K_R are dependent on pH as the reactivation process is essentially ionic in nature, involving a protonated form of the inhibited enzyme and tetrapolar ion of the reactivator. Tetrapolar ion concentrations were computed with $pK_{a1} = 7{\cdot}95$ and $pK_{a2} = 8{\cdot}88$. The pK_a values were obtained potentiometrically[1] and refer to the oxime groups dissociation.

The $pK_R^{\pm}$ obtained in this way are those for the reaction $EIH^+ + R^{\pm} \rightleftharpoons EIHR$. The effect of pH on $pK_R^{\pm}$ is shown in Fig. 1. The inflection point at 7·0 in Fig. 1 is taken as the dissociation constant for the inhibited enzyme EIH^+ and is designated as pK_{EIH+}.[6] In the pH region from 7·0 to 8·0 the dissociation of the intermediate complex gives no change of charge and zero slope was observed. At pH lower than pK_{EIH+} the change of two units charge gives rise to a + 2 slope.

The pH optimum can be expressed as midway between pK_a of the reactivator and pK_{EIH+} of the inhibited enzyme[7]

$$pH_{opt} = \frac{pK_a + pK_{EIH+}}{2}.$$

The present values 7·0 for pK_{EIH+} and 7·95 for pK_a (when pK_{a2} value is neglected) give the value 7·5 for pH_{opt}. The dependence of bimolecular rate constant k_r upon the pH is shown in Fig. 2. This

treatment gives the location of pH optimum at 7·25. The observed value of pH_{opt} is lower than the calculated one by 0·25 unit. The small differences between observed and calculated values of pH_{opt} for oxime reactivation of organophosphate inhibited acetylcholinesterase were observed also by other authors.[7,8]

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Effect of 3-methylcholanthrene induction on the carbon tetrachloride-induced changes in rat hepatic microsomal enzyme system

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THE WELL known impairing effects of carbon tetrachloride (CCl_4) on oxidative enzymes in hepatic microsomes[1–3] are probably due to a decrease in microsomal heme components.[4–6] It is now believed that these effects are mediated by an active metabolite of CCl_4.[7–10] In accord with this view, treatment of rats with phenobarbital (PB) enhances the impairing effects of CCl_4 on ethylmorphine (EM) *N*-demethylation, the destruction of cytochrome P-450[4] and the metabolism of CCl_4[11] and decreases the LD_{50} of the toxicant.[11]

While treatment of rats with PB increases the metabolism of a wide variety of drugs presumably by increasing the amount of the type of cytochrome P-450 already present in hepatic microsomes,[12] treatment with 3-methylcholanthrene (3-MC) enhances the metabolism of relatively few compounds,[13] presumably by inducing the formation of a different kind of cytochrome P-450 called cytochrome P-448.[12] It therefore seemed important to study the effects of CCl_4 in 3-MC treated rats.

In these studies, 3-MC dissolved in corn oil was administered, i.p., in a dose of 20 mg/kg three times at 12-hr intervals to 180-g male Sprague–Dawley rats from Hormone Assay (Chicago, Ill.). Control rats received the same volume of corn oil. Forty-eight hr after the last injection the animals, which had been fasted overnight, received 2·5 ml of CCl_4/kg orally, while controls received the same volume of saline. At different time intervals thereafter the rats were decapitated, and hepatic microsomes were isolated as previously described.[6] Protein concentrations were determined by the biuret method.[14] The amounts of cytochrome P-450, cytochrome P-420 and cytochrome b_5 were determined spectrophotometrically as described by Omura and Sato.[15] The amount of total heme was estimated as described by Falk.[16] An Aminco-Chance dual wavelength spectrophotometer was used for these determinations. EM metabolism was determined in incubation mixtures, consisting of 5 mM $MgCl_2$, 12 mM glucose 6-phosphate, 1·00 U of glucose 6-phosphate dehydrogenase per 3 ml, 0·33 mM nicotinamide adenine dinucleotide phosphate (NADP), 50 mM tris buffer (pH 7·4), 5 mM EM and