Finally, the current interest in cyclic nucleotide phosphodiesterase and the well known facts that imidazole stimulates and theophylline inhibits this enzyme will undoubtedly lead to an examination of imidazole derivatives and similar compounds in a search for inhibitors.[15] Pharmacological studies of such compounds are likely to center on their ability to potentiate the action of drugs and hormones which are believed to act via cyclic AMP.[16] In view of our results, it would be prudent to rule out effects on drug metabolism when considering the mode of action of imidazole derivatives in studies of that nature.

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

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WE HAVE recently studied the interaction of α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide with isopropyl-methylphosphonylated acetylcholinesterase.[1,2] This compound is a strong reactivator of isopropyl-methylphosphonylated acetylcholinesterase.[1]

We have previously studied the influence of pH on the reactivating effect of this oxime.[2] In this study, the influence of temperature on reactivating effect, with the use of isopropyl-methylphosphonylated bovine erythrocyte acetylcholinesterase is described.

α,ω-Bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide was prepared as reported previously.[1]

Acetylcholinesterase used in this work was prepared from bovine erythrocytes.[3] The specific activity of the lyophilized enzyme preparate was 0·265 μm of acetylcholine per min per milligram at 25°

and pH 8·0. The K_m of 0·58 mM for acetylcholine as substrate was estimated. Acetylcholine iodide was obtained from Lachema, Brno.

Acetylcholinesterase activity was measured electrometrically according to Michel[4] by a method with the direct recording.[5] A 2·76 mM solution of acetylcholine iodide served as substrate. Estimations were carried out in Michel phosphate–barbital buffer[4] (pH 8·0) isotonized by sodium chloride [0·9% (w/v)] at 25°. The described apparatus[5] was employed also for all kinetic measurements.

Inhibition of acetylcholinesterase was performed at 25° by 30 min preincubation of enzyme with 5 nM isopropyl-methylphosphonofluoridate. The reactivation was carried out over the range of α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide concentration from 0·01 to 0·1 mM and at pH 8·0 in a 20-mM sodium phosphate buffer isotonized by sodium chloride over a temperature range between 5 and 40°. Temperature was maintained at constant level (±0·1°) by means of a standard temperature apparatus (Thermoelectric thermostat type WP 760 08, Tesla Lanskroun).

For each temperature set of reactivation experiments the apparent first order rate constants k_{app} were calculated.[1,6] When the reciprocals of k_{app} vs molar concentration of reactivator were plotted, a straight line which did not pass through the origin was obtained. The slopes of the lines give $1/k_r$, the intercepts on the ordinates give $1/k_R$, and the intercepts on the abscisses give $1/K_R$. The lines were computed (MINSK-22 computer) by a least squares method.[7]

TABLE 1. KINETIC PARAMETERS OF REACTIVATION OF ISOPROPYL-METHYL-PHOSPHONYLATED ACETYLCHOLINESTERASE (EI) BY α,ω-BIS-(4-HYDROXY-IMINOMETHYLPYRIDINIUM)-2-TRANS-BUTENE DIBROMIDE (R) AT DIFFERENT TEMPERATURES

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

$$EI + R \xrightarrow{k_r} E + P$$

Temperature (°C)	$k_R \pm$ S.E. (min^{-1})	$K_R \pm$ S.E. (μM)	$k_r \pm$ S.E. ($10^3 \times M^{-1}$ min^{-1})
5·2	0·069 ± 0·008	45·4 ± 2·6	1·42 ± 0·16
11·4	0·088 ± 0·005	35·2 ± 2·0	2·48 ± 0·13
15·5	0·110 ± 0·006	30·4 ± 1·8	3·67 ± 0·10
20·4	0·129 ± 0·012	23·0 ± 2·3	5·60 ± 0·58
25·6	0·152 ± 0·010	15·8 ± 1·0	9·76 ± 0·62
30·6	0·189 ± 0·006	14·8 ± 1·1	12·68 ± 0·46
36·4	0·236 ± 0·013	14·5 ± 1·5	16·35 ± 0·65
40·8	0·281 ± 0·022	15·9 ± 1·7	17·72 ± 0·84

The computed kinetic constants for all temperature used are shown in Table 1. The values of the standard free energies of binding at each temperature were calculated from the equation

$$\Delta G^\circ = -RT \ln K_R$$

and the standard error (S.E.) was found from the equation

$$\text{S.E.}\ (\Delta G^\circ) = RT \frac{\text{S.E.}\ (K_R)}{K_R}$$

as indicated by Wilkinson.[8] This values are given in Table 2.

The standard enthalpy of binding is given by

$$\log K_R = -\frac{\Delta H^\circ}{2{\cdot}303\ R} \times \frac{1}{T} + \text{constant.}$$

The plot of log K_R against $1/T$ will give a line with a slope of $\Delta H^\circ/2{\cdot}303\ R$. As shown in Fig. 1 the dependence of log K_R on temperature is significant up to transition temperature 27°. Over this

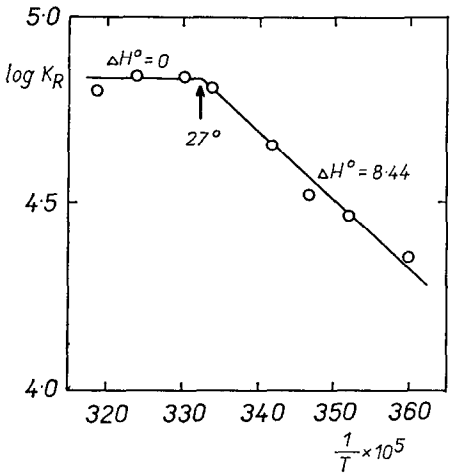

FIG. 1. The effect of temperature on the dissociation constant K_R for the reactivation of isopropylmethylphosphonylated acetylcholinesterase by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide. ΔH° given in kcal/mole.

point is no significant temperature dependence of enthalpy change. The values of ΔH° were calculated from least squares treatment. The standard error of ΔH° was calculated from the equation[8]

$$\text{S.E.}\ (\Delta H^\circ) = 2{\cdot}303\ R \times \text{S.E.}\ (\text{slope}).$$

This value of enthalpy change was obtained: $\Delta H^\circ = 8{\cdot}44 \pm 0{\cdot}80$ kcal/mole. The values for the standard entropy changes were calculated by substituting ΔH° and ΔG° in the equation

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T}$$

and are given in Table 2. It is of interest to note that at physiological temperatures the lower entropy predominates.

TABLE 2. THERMODYNAMIC PARAMETERS OF REACTIVATION OF ISOPROPYLMETHYLPHOSPHONYLATED ACETYLCHOLINESTERASE BY α,ω-BIS-(4-HYDROXYIMINOMETHYLPYRIDINIUM)-2-TRANS-BUTENE DIBROMIDE

Temperature (°C)	$\Delta G^\circ \pm$ S.E. (cal/mole)	ΔS° (e.u.)
5·2	−5520 ± 93	+4·95
11·4	−5800 ± 74	+4·95
15·5	−5980 ± 35	+4·95
20·4	−6230 ± 134	+4·95
25·6	−6550 ± 86	+4·95
30·6	−6700 ± 103	+2·20
36·4	−6800 ± 147	+2·21
40·8	−7000 ± 156	+2·20

Free energies and entropies of reactivation given in calories and standard entropy units, respectively. $\Delta H^\circ = 8{\cdot}44 \pm 0{\cdot}80$ kcal/mole.

The effect of temperature on the bimolecular rate constant k_r and the decomposition rate constant k_R, respectively, is shown in Fig. 2. The activation energy, E_a, was obtained from the slopes of both lines by an unweighted least squares analysis. E_a for decomposition of complex EIR is 6·36 ± 0·27 kcal/mole. The activation energy computed from temperature dependence of bimolecular rate constant k_r is 11·9 ± 1·2 kcal/mole. This value is comparable with activation energies of reactivation

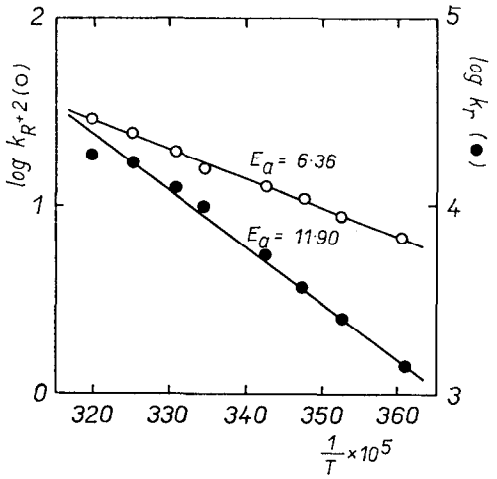

FIG. 2. An Arrhenius plot for the reactivation of isopropylmethylphosphonylated acetylcholinesterase by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide. Open circles: the dependence of decomposition rate constant k_R on temperature. Full circles: the dependence of bimolecular rate constant k_r on temperature. E_a given in kcal/mole.

of isopropyl-methylphosphonofluoridate (11·5 kcal/mole) and tetraethylpyrophosphate (11·4 kcal/mole) inhibited erythrocyte acetylcholinesterase by diisonitrosoacetone.[9] Similar values were obtained by Wang and Braid[6] for reactivation of diethylphosphoryl human serum cholinesterase by 2-hydroxyiminomethylpyridinium methiodide (12·8 kcal/mole) and isonitrosoacetophenone (15·6 kcal/mole), respectively. Wilson[10] found the activation energy of reactivation tetraethylpyrophosphate inhibited erythrocyte acetylcholinesterase by choline (14·5 kcal/mole) and hydroxylamine (30 kcal/mole), respectively.

While the entropy of activation for the reactivation of isopropyl-methylphosphonofluoridate inhibited acetylcholinesterase by α-ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide is 2·20 and 4·95 e.u., respectively, the ΔS° for the reactivation of tetraethylpyrophosphate inhibited acetylcholinesterase by hydroxylamine is −37 e.u. and thus is very high.[10]

The comparison of our results with data in the literature is very difficult as the published thermodynamic constants of reactivation of phosphorylated cholinesterases are very poor. To elucidate the influence of reactivator structure on thermodynamic parameters of reactivation, further investigations are required.

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Effect of 2,3,7,8-tetrachlorodibenzo-1,4-dioxin on drug metabolism in the rat

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2,3,7,8-TETRACHLORODIBENZO-1,4-dioxin (dioxin),* a potential contaminant of the herbicide 2,4,5-trichlorophenoxyacetic acid, has been shown to be very toxic to various animal species[1–3] and the probable cause of industrial poisoning following accidents in 2,4,5-trichlorophenol production.[1,2,4] It also possesses foeticidal and teratogenic properties in rats and mice.[5,6] Recently French workers[7] have demonstrated that dioxin is a powerful stimulator of the enzymes that detoxify zoxazolamine in the rat; a dose of dioxin as low as 50 μg/kg being capable of shortening the zoxazolamine paralysis time by 90 per cent after 1 day. The work described here confirms this result and shows that dioxin also prolongs the action of hexobarbitone in the rat.

Male rats of the albino Porton strain weighing 180–200 g (7–8 weeks old) and with access to diet 41B and water *ad lib.* were given a single oral dose of dioxin (200 μg/kg, 100 μg/ml in Arachis oil). After either 1 or 3 days the duration of the paralysis induced by zoxazolamine hydrochloride (100 mg/kg i.p.) was measured and found to be reduced by over 54 per cent from that of oil-dosed controls (Table 1). The quantitative difference between these results and those of Buu-Hoi *et al.*[7] is most probably due to variations in the age and strain of rats used; although they quote an age of 3 months, the weight of their rats (100 g) suggests a younger animal in which the basal level of the liver microsomal oxidases would be much lower.[8]

TABLE 1. ZOXAZOLAMINE PARALYSIS TIME (MIN) OF MALE RATS TREATED WITH DIOXIN

Days after dioxin	Treated	Controls	P
1	22·0 ± 3·9 (6)	48·1 ± 8·5 (6)	0·015
3	16·9 ± 1·1 (7)	65·6 ± 6·5 (8)	0·00031

Values quoted are the mean ±S.E., the number of observations in parentheses. Significance is based on Wilcoxon's ranking test. Details of dosages are given in the text.

The zoxazolamine paralysis time is an *in vivo* measure of the activity of liver enzymes which oxidize certain aromatic substrates.[8] Since the level of these enzymes can be raised specifically by compounds such as 3-methylcholanthrene without affecting other liver oxygenases, the effect of dioxin on the hydroxylase which metabolizes hexobarbitone was determined. Male and female rats weighing 180–200 g (♂ 7–8 and ♀ 8–11 weeks old) were given a single oral dose of either dioxin (200 μg/kg, 100 μg/ml in dimethyl sulphoxide) or an equivalent volume of dimethyl sulphoxide. The sleeping time of the animals following an intraperitoneal injection of hexobarbitone sodium (♂ 150 mg/kg; ♀ 75 mg/kg) was measured either 1 or 3 days after dosing. With both sexes at 1 day there was a significant prolongation of the sleeping time, which at 3 days was more than double that of the controls (Table 2).

* Abbreviations used: 5-*cyclo*hex-1′-enyl-1,5-dimethylbarbituric acid, hexobarbitone; 2,3,7,8-tetrachlorodibenzo-1,4-dioxin, dioxin; 2-amino-5-chlorobenzoxazole, zoxazolamine.