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On the Reversible Enzyme Inhibition by Tetraethyl Pyrophosphate (TEPP)

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Much attention has been brought to compounds with strong inhibiting action on cholinesterases. One type of these "anticholinesterases" are reversible inhibitors (*e. g.*, physostigmine, prostigmine). The second type of inhibitors are represented by, amongst others, the organophosphorus compounds which act as powerful inhibitors (see the review by Koelle and Gilman [1]). These latter compounds have been regarded as irreversible inhibitors. Neither dilution nor dialysis can restore the inactivated cholinesterase activity. However, two observations have been published recently [2,3] which demonstrate the *in vitro* reversibility of the tetraethyl pyrophosphate (TEPP) inhibition in some cases. Earlier, an observation of this kind was made by Grob and Harvey [4] who showed that the *in vivo* inhibition of erythrocyte acetylcholinesterase by TEPP was reversible for the first 24 hours. The reversible TEPP inhibition *in vitro* was demonstrated by Hobbiger [2] with the esterases of erythrocytes, brain, intestine, and salivary glands; human serum was exceptional. The observation of Wilson [3] was made with purified acetylcholinesterase from the electric tissue of *Electrophorus*. The enzyme, inhibited by TEPP, was reactivated by reactions of the enzyme-inhibitor complex with water or more rapidly with choline or hydroxylamine.

A similar demonstration of the reversibility of TEPP inhibition had been made in this laboratory when these two papers [2,3] came to our knowledge. The experiments reported here have been carried out with esterases of various sources; special attention has been given to the cobra venom acetylcholinesterase.

METHODS AND MATERIAL

The esterase activity was measured with the Warburg manometric technique previously described in detail [5]. The enzyme activity is expressed in b_{30} values, *i. e.*, the amount of CO_2 in μl evolved during 30 minutes minus the corresponding value for non-enzymic hydrolysis. All measurements were made at 25° C.

In most experiments reported in the following, the enzyme source was a dried and crystallised preparation of cobra venom *(Naja naja)*. The venom was dissolved in bicarbonate-Ringer's solution. In this solution the esterase is stable for months [6]. Human plasma and human erythrocytes and also *Helix* blood have been employed in some experiments.

Acetylcholine chloride was used as substrate; the final concentration in the reaction mixture was 3.30×10^{-3} *M*. Tetraethyl pyrophosphate (TEPP) was a pure sample *.

RESULTS

Various enzyme preparations were incubated with TEPP at pH 7.4. The concentration of the inhibitor was chosen to give about 90 % inhibition. Cholinesterases prepared from the following sources were tested: human plasma, human erythrocytes, cobra venom, and *Helix* blood.

In most of the experiments the TEPP treated enzyme solutions together with the controls were kept in the refrigerator (4.5° C). The esterase activity was measured after one hour and then after various lengths of time, continuing for 60 to 80 days. The results are illustrated in Table 1 and Figs. 1 and 2.

Table 1. Reactivation of cholinesterase from various sources after inhibition by TEPP. Values refer to per cent of control for each determination.

Days of incubation with TEPP	Human plasma	Human erythrocytes	Cobra venom	*Helix* blood
1/24	5.5	8	4	3.5
1	7	10	4	3.5
5	7.5	14	5	7
10	—	—	21	12
21	12	21	51	17
49	25	29	68	22.5
59	27	39	—	—
77	—	—	71	25

* TEPP was synthesised and kindly placed at our disposal by Dr. Bo Holmstedt, Research Institute of National Defence, Department I, Ulriksdal, Sweden.

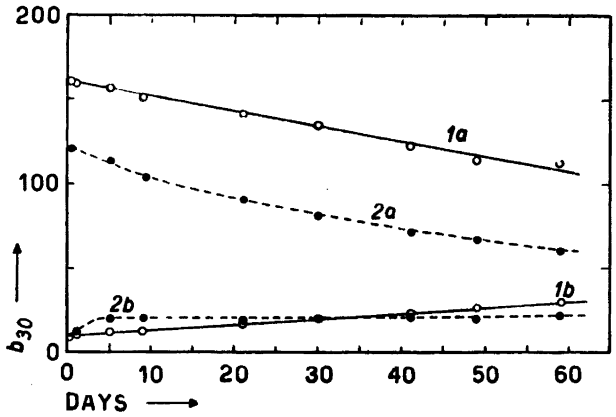

Fig. 1. Reactivation of human plasma cholinesterase (1) and human erythrocyte acetylcholinesterase (2) after inhibition by TEPP. Incubation at 4.5° C. a: controls; b: TEPP treated solutions.

Reactivation of esterase activity after inhibition by TEPP was observed in all cases studied. The recovery process was fastest with solutions of cobra venom, slowest with human plasma and *Helix* blood. For example, in case of cobra venom little reactivation occurred during the first 5 days, but after 3 weeks the activity was 50 %, after 11 weeks 70 % of control. Erythrocyte esterase activity was restored to about 20 % of control after 3 weeks.

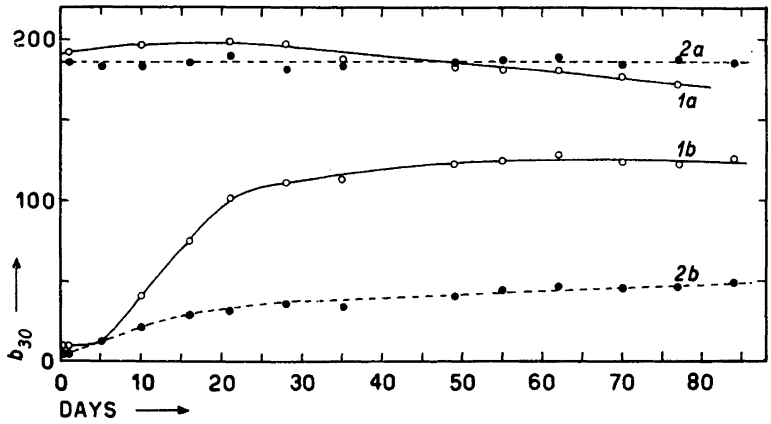

Fig. 2. Reactivation of acetylcholinesterase of cobra venom (1) and Helix blood (2) after inhibition by TEPP. Incubation at 4.5° C. a: controls; b: TEPP treated solutions.

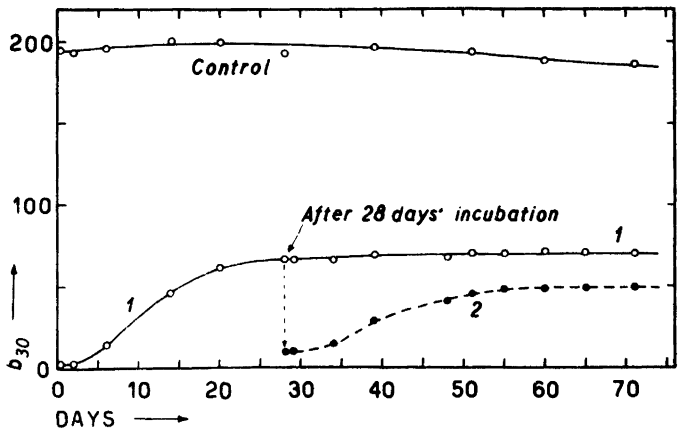

Fig. 3. Reactivation of cobra venom acetylcholinesterase after inhibition by TEPP. Incubation at 4.5° C. After a 28 day incubation period with TEPP this solution (1) was again treated with TEPP (2).

Fig. 3 also illustrates the reversibility of the TEPP inhibition of cobra venom acetylcholinesterase. After a 28 day incubation period, the TEPP treated solution, the activity of which was 33% of control, was divided into two parts. One part was treated with another portion of TEPP to reduce the activity to about 5%, the other part was diluted with bicarbonate solution and used as a second control. The esterase activity was again found to be restored in the solution which had been treated twice with TEPP. After another 30 days the activity was 72% of the second control, 26% of the first control.

The reactivation of cholinesterases after inhibition by TEPP may be due to dissociation of the enzyme-inhibitor complex or to a destruction of inhibitor. The complex, most probably, is a phosphorylated enzyme. In the reactivation processes described above it appears likely that water reacts with this phosphorylated enzyme in the same way as it does with the acetylated esterase in the acetylcholine hydrolysis. The former process is a slow one in comparison with the latter. The rate of reactivation after TEPP inhibition is measurable, for other organophosphorus compounds tested it is too slow to be detected. This is the case with DFP (di*iso*propyl fluorophosphate) and Tabun (dimethylethoxy-phosphoryl cyanide) [7].

It has been reported by Wilson *et al.* [3,8] that acetylated acetylcholinesterase (of electric tissue) reacts with hydroxylamine in a way similar to the reaction with water. It was demonstrated later that hydroxylamine also reacts with the phosphorylated enzyme. At that time the present authors had performed experiments with hydroxylamine which was shown to have a remark-

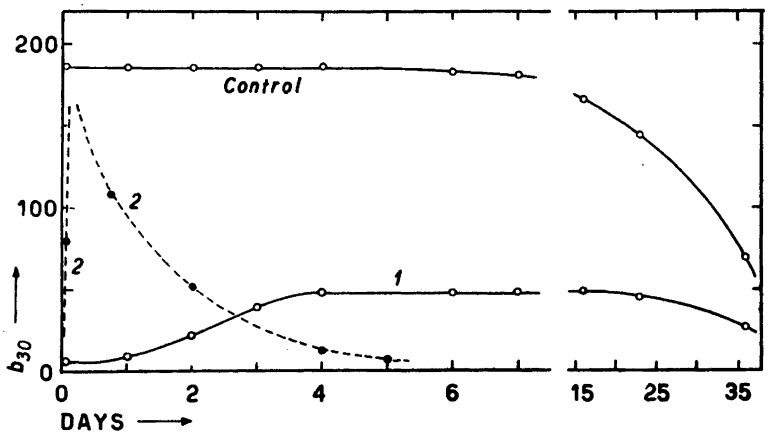

Fig. 4. Reactivation of cobra venom acetylcholinesterase after inhibition by TEPP. The effect of hydroxylamine on this process. The solutions were kept at 25° C. 1) No hydroxylamine; 2) Hydroxylamine added after 10 minutes' incubation, 0.05 M NH_2OH in the incubation mixture.

able effect on the reaction process after inhibition by TEPP. A typical experiment carried out with cobra venom acetylcholinesterase is shown in Fig. 4. The esterase was incubated for 10 minutes with TEPP, and hydroxylamine was then added to give 0.05 *M*. The reaction between TEPP treated enzyme and hydroxylamine of that concentration is too rapid to be measured accurately. In about two hours the activity was restored almost completely.

After restoration of activity another process begins. This is a comparatively slow reaction between the reactivated enzyme and phosphorylated hydroxylamine. In this process the esterase is again inhibited. The inhibition observed was 50% after about one hour, it was almost complete after 5 days. The difference in reaction rates between these two processes, dephosphorylation and subsequent inhibition, has made it possible to detect reactivation of TEPP inhibited enzyme.

Other compounds also have been tested for their influence on the reactivation process after inhibition by TEPP. Up to the present, cobra venom has been used in these experiments and pH of the incubation mixtures has been 7.4. Choline, arginine, and creatinine have been tested so far. Arginine at a concentration of 10^{-3} *M* has practically no influence on the rate of reactivation. Creatinine at a concentration of 7×10^{-3} *M* seems to react with phosphorylated enzyme and thereby the esterase activity is restored to a certain degree more rapidly than in the absence of this reagent.

Choline and all choline derivatives investigated are competitive and reversible inhibitors of cholinesterases [5]. Choline reacts with TEPP inhibited esterase demonstrated with 1.43×10^{-2} *M* choline chloride. The activity was restored to 25% of control after 2 days (for comparison with control, see Fig. 2). Higher choline concentrations would give better results, as was shown by Wilson [3] with the electric tissue esterase.

DISCUSSION

The inhibiting action of organophosphorus compounds on cholinesterases has been regarded an irreversible process until quite recently. *In vitro* experiments, however, have demonstrated conclusively that esterases inhibited by some of these compounds (see below) are reactivated although this process under ordinary conditions is comparatively slow. Thus, TEPP inhibited cholinesterases of various sources were reactivated; these sources were erythrocytes, brain, salivary glands [2], and electric tissue [3]. In the present paper reactivation was demonstrated also with cobra venom and *Helix* blood, both materials containing acetylcholinesterase. A small reactivation was also observed with the esterase of human plasma which was an exception in Hobbiger's series. TEPP has been the only phosphate investigated that renders the inhibited enzyme reversible; however, among the newly discovered "dialkylphosphostigmines" the lower homologoues are also reversible inhibitors [11] (see below). The inhibition by DFP *in vitro* is irreversible or its reversibility is too slow a process to be detected. The inhibition by Tabun also is not found to be reversible.

Various factors influence the degree of inhibition *in vitro* by these "irreversible anticholinesterases" (see review by Whittaker [9]). Most certainly the inhibition can be interpreted as a phosphorylation process, in which the enzyme is not destroyed. A direct proof for this has been produced by Jansen *et al.*[10] with α-chymotrypsin inhibition by ^{32}P labelled DFP. A similar process is most probably valid for cholinesterases. The phosphorylation is a comparatively slow reaction. This first reaction is supposed to be reversible and is dependent upon the various reactive groups around the phosphoryl group, $\rangle P = O \leftrightarrow \rangle P^+ \rightarrow O^-$. We do not know exactly the distribution of electric charges in this linkage, but it is certain that the phosphorus atom is relatively positive. The more positive this atom is, the greater reactivity is possessed by the compound. The phosphorus atom in DFP is more electrophilic than in TEPP which explains the difference in reactivation after enzyme inhibition. Wilson [3] has recently discussed these problems from a similar point of view.

In the reactivation process after inhibition by TEPP, described above, the inhibitor is not regenerated. Therefore, after the initial complex has been formed one part of the inhibitor is supposed to set free. This secondarily formed complex is now susceptible to hydrolytic dissociation or dephosphorylation. In case of dialkylphosphates, Burgen and Hobbiger [11] have recently found evidence for the theory that the duration of action of these compounds is determined by the dialkylphosphoryl residue.

No doubt, there is a great difference between the chemical structures of the classical "anticholinesterases", physostigmine and prostigmine, on one hand, and the "irreversible inhibitors", the organophosphorus compounds, on the other. The high affinity for cholinesterases, common to both these groups of inhibitors, may be due to similarity in electronic structures of the carbonyl group (in urethanes) and the phosphoryl group. As the phosphorus atom in the phosphoryl compounds, the carbon atom in the urethanes is also electrophilic. Both groups react with the active centre II [12] (the esteratic site, according to Wilson *et al.*). If the inhibitor molecule contains, in addition to one of these electrophilic groups, a positively charged atom, *e.g.*, a quaternary nitrogen atom as in prostigmine or the recently synthesised "dialkylphosphostigmines" [11], there is a two-point contact, now also with the active centre I (the anionic site). This latter combination is reversible. The inhibitor molecule of the complex formed is not altered as in the case of an one-point contact at the centre II. This probably explains the difference in kinetic behaviour of the various "anticholinesterases".

SUMMARY

Cholinesterases inhibited by tetraethyl pyrophosphate (TEPP) are reactivated slowly in vitro. The reversible inhibition by TEPP has been demonstrated with human plasma, human erythrocytes, cobra venom, and *Helix* blood. The rates of this recovery process are different for various enzyme preparations. The highest rate was obtained with cobra venom acetylcholinesterase, the lowest with human plasma and *Helix* blood.

The enzyme-inhibitor complex reacts with hydroxylamine to yield reactivated enzyme and hydroxylamine phosphate, which in its turn is a slowly reacting inhibitor of the enzyme.

The effects of choline, arginine, and creatinine respectively on the recovery process are shortly reported.

The mechanism of action of enzyme inhibition by organophosphorus compounds is discussed from a general point of view.

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