Anticholinesterase Activity of Triethyl Phosphate Resulting from an Impurity

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The apparent anticholinesterase potency of commercially available triethyl phosphate is essentially all due to the presence of an impurity detectable by its instability. The rate of hydrolysis of this inhibitor was the same as that of tetraethyl pyrophosphate. Distillation of triethyl phosphate at atmospheric pressure resulted in a sixfold increase in inhibitor concentration. Divergent potencies given in the literature for the inhibition of cholinesterase by triethyl phosphate suggest such contamination may be common and, thus, a source of potential error in studies attempting to elucidate the relationship between the structure of alkyl phosphates and their anticholinesterase properties. The rate of reaction of triethyl phosphate with rat brain cholinesterase, expressed as the bimolecular rate constant, is 0.42 liter mole^{-1} min.$^{-1}$.

Triethyl phosphate (TEP) has been of interest in systematic investigations correlating the structure of trialkyl phosphates to their anticholinesterase potency. While TEP is regarded as possessing essentially no inhibitory activity compared to other derivatives of diethyl phosphate which are the basis of many organophosphate pesticides, significant but divergent values for its potency have been reported (Cheymol et al., 1960; Bracha and O'Brien, 1968). Additional interest in this compound involves its potential use as a whipping aid in commercially pasteurized egg whites (Cunningham et al., 1967). In the course of in vivo toxicity studies with TEP, we found considerable in vitro inhibitory activity. It seemed likely that at least a large portion of the observed inhibition might be the result of potent impurities. Especially suspect was tetraethyl pyrophosphate (TEPP) since the starting materials commonly used in the synthesis of trialkyl phosphates, such as phosphorus oxychloride or dialkyl chlorophosphates, also react with trialkyl phosphates to produce corresponding pyrophosphate derivatives (Metcalf, 1955). In this regard, diethyl phenyl phosphate and several of its analogs derived from diethylphosphoric acid were all found to possess such an impurity (Aldridge and Davison, 1952).

Reported here are the results of an investigation into the stability of the apparent inhibitory potency of TEP toward hydrolysis and the determination of a new, low value for the anticholinesterase activity of TEP.

EXPERIMENTAL

TEP was subjected to conditions of hydrolysis consisting of heating an aqueous solution of the compound (100 mg. per ml.) in 0.1N HCl at 100° C. The effect of this treatment on anticholinesterase activity was determined on samples removed from the heating bath at 0, 30, 60, and 120 minutes, immediately neutralized with 0.1N NaOH, and diluted with water to concentrations found in preliminary experiments which would span that, causing 50% enzyme inhibition. For comparison, a similar experiment was conducted with TEPP (1.5 mg. per ml.). Inhibitory potency toward cholinesterase was measured by reacting 0.1 ml. of properly diluted sample with 0.4 ml. of enzyme for 1 hour at 25° C., followed by assay of residual enzyme activity using the ferric chloride–hydroxylamine method as described by Fleisher et al. (1955) with acetylcholine as the substrate. The source of enzyme was 3% (w./v.) rat brain homogenate in distilled water.

The stability of TEP toward the above hydrolytic treatment

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was determined with TEP-1,2-^{14}C (5 mg. per ml., 2 × 10^4 d.p.m. per mg.). Samples were removed from the heating bath at intervals up to 2 hours, neutralized, and partitioned with chloroform. The first-order rate constant of hydrolysis was calculated from the rate of increase of ^{14}C in the aqueous phase after making small corrections for the partitioning of ethanol and unreacted TEP. The partition coefficients (aqueous: chloroform) at 23° C., also determined with radioactive samples, were 6.9 for ethanol and 5.9 × 10^{-3} for TEP. Radioactivity was measured by liquid scintillation counting techniques.

Bimolecular rate constants for the reaction of enzyme with inhibitor were calculated for comparison of data in the literature using the relationship: $k_i = 0.695/I_{50} t$, where I_{50} is the molar concentration giving 50% inhibition and t is time (O'Brien, 1960). Triethyl phosphate, Eastman Organic Chemicals, was redistilled at atmospheric pressure, b.p. 215 to 217° C. Tetraethyl pyrophosphate (40%) was obtained from K & K Laboratories, and TEP-1,2-^{14}C from the New England Nuclear Corp.

RESULTS AND DISCUSSION

The relative stability of TEP in 0.1N acid and 100° C. reported by Masse and Domange (1960) was confirmed in our laboratory, where a first-order rate constant for hydrolysis equal to 4.6 × 10^{-4} min.$^{-1}$ was obtained. The effect of this hydrolytic treatment on the inhibitory activity of solutions of TEP (redistilled) and TEPP is shown in Figure 1. Increasing periods of treatment brought about a marked and progressive increase in the relative volume of both solutions required to produce 50% enzyme inhibition, even though in the case of the TEP sample, TEP would be only 5.4% hydrolyzed after 2 hours.

A conventional plot of log inhibitor concentration with time (Figure 2) shows that the rate of hydrolysis of inhibitor in both samples may be regarded as following the sum of two first-order reactions resulting from the presence of two inhibitors, thus:

$$\log \% I_{50} (\text{apparent}) = \log\left[\frac{I_{0_1}}{I_{50_1}} e^{-k_1 t} + \frac{I_{0_2}}{I_{50_2}} e^{-k_2 t}\right] + 2$$

where I_{50} is the molar inhibitor concentration giving 50% enzyme inhibition, I_0 is the molar inhibitor concentration at zero time, k is the hydrolysis constant of inhibitor, and t is time. Correcting the rate of disappearance of inhibitor in both samples for the contribution of the more stable inhibitor, a contribution that was negligible for the first 30 minutes, yields a rate of inhibitor hydrolysis in TEP identical to that of TEPP

Table I. Anticholinesterase Potency of Triethyl Phosphate

Source of Data	$k_i{}^a$
Cheymol et al. (1960)	300[b]
Bracha and O'Brien (1968)	<7.0[c]
Present Study	
Sample 1 (redistilled)	
Apparent	120
After treatment	0.42
Sample 2	
(Undistilled) apparent	20
(Redistilled) apparent	124

[a] Bimolecular rate constant for reaction of TEP with cholinesterase, liter mole^{-1} min.$^{-1}$ at 25° C.
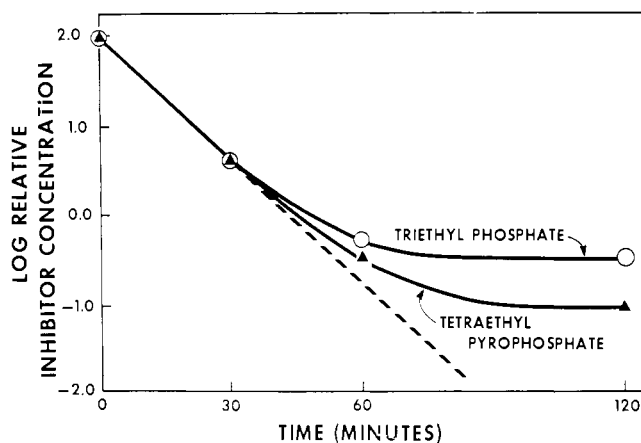[b] Based on 15-minute reaction time for inhibitor with enzyme, Enzyme source not specified.
[c] Determined on bovine red blood cell acetylcholinesterase.
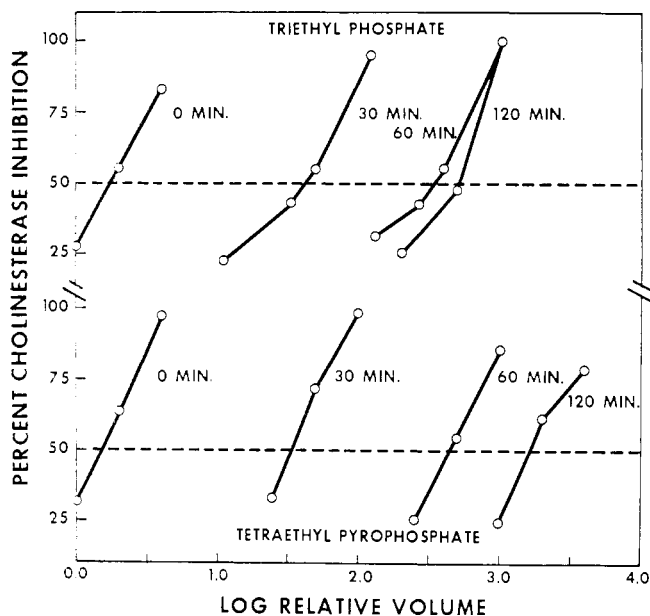
Figure 1. Effect of hydrolytic treatment on anticholinesterase potency of TEP and TEPP after various times

Figure 2. Rate of hydrolysis of inhibitor in TEP and TEPP

Relative inhibitor concentration was calculated from reciprocal of relative volumes producing 50% inactivation as derived from Figure 1

A second sample of TEP (Eastman Organic Chemicals) was analyzed before and after distillation. As shown in Table I, distillation at atmospheric pressure brought about approximately a sixfold increase in inhibitory activity. The apparent production of trace amounts of TEPP under these conditions is in accord with the thermal decomposition of TEPP to yield TEP and monoethyl phosphate which takes place at 200° C. and has been reported to be reversible (Kosolapoff, 1950).

The contamination of TEP presumably by TEPP and of other alkyl phosphates by corresponding pyrophosphates is suggested to be sufficiently probable as to provide a potential source of error in studies correlating structure to anticholinesterase activity. Thus, routine screening for the presence of more than one inhibitor in such compounds would seem desirable in work of this nature.

and equal to 0.106 min.$^{-1}$ (broken line in Figure 2). After 120 minutes the remaining cholinesterase inhibitor activity can be entirely attributed to TEP or, in the case of the TEPP sample, to impurities of extremely low potency. This yields a bimolecular rate constant for the reaction of TEP with cholinesterase equal to 0.42 liter mole^{-1} min.$^{-1}$ (I_{50} = 2.8 × 10^{-2}M). The considerably greater potency measured by Cheymol et al. suggests the presence of similar contamination (Table I). Under conditions of our assay, the concentration of TEPP giving 50% inhibition was 7.2 × 10^{-9} M. Thus, before hydrolytic treatment, the contamination of TEP, calculated as TEPP, was only 0.012% by weight and was responsible for all of the observed anticholinesterase activity.

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