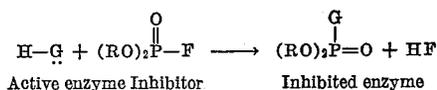


anism of inhibition and of reactivation is very closely related to the mechanism of enzymic hydrolysis. This enzyme contains two sites, (i) an anionic site which contributes to the catalytic activity by binding and orienting molecules containing substituted ammonium structures, and (ii) an esteratic site which interacts with the ester function and is primarily responsible for the hydrolytic activity. During the hydrolysis of a carboxylic ester a basic group in the esteratic site is acylated to form an acyl-enzyme as intermediate. Acetyl-enzyme (from acetate-esters or anhydrides) rapidly reacts with water to produce acetic acid and to regenerate the free and active enzyme. The alkyl phosphate inhibitors react with the same basic group to form a dialkyl phosphoryl enzyme which however reacts only very slowly with water.

The inhibitory reaction is illustrated for a fluorophosphate:

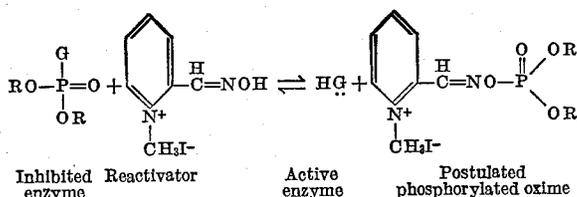


where H—G represents the esteratic site containing an acidic group (H) and a basic group (...). Theory predicts that nucleophilic reagents should dephosphorylate the enzyme and thus restore its activity. When R=ethyl (inhibitor=diethyl fluorophosphate or tetraethyl pyrophosphate (TEPP)) reactivation is readily accomplished by a large number of suitable compounds. When R=isopropyl (inhibitor=diisopropyl fluorophosphate (DFP)) reactivation is more difficult.

Experiments show that the anionic site survives the inhibition of the enzyme and can contribute to the reactivation process. Therefore a very good reactivator might be produced by combining in the same molecule an intrinsically good functional group and a suitably located quaternary ammonium structure. These studies also show that the intrinsic activity could be augmented by a pyridine nucleus.

Therefore, 2-pyridine aldoxime methiodide was prepared in order to evaluate the effect of introducing a quaternary structure. It was found to be extremely active in reactivating the inhibited enzyme formed with two representative inhibitors, tetraethylpyrophosphate (TEPP) and diisopropyl fluorophosphate (DFP).

The results are presented in Table IA. From these results it appeared that measurements with TEPP might not be rate measurements but rather the extent of reactivation achieved at equilibrium of the reaction:



To test this possibility the inhibited enzyme was diluted 400 times (instead of 7.5 times) before the reactivator was added. Under these circumstances the postulated phosphorylated oxime should be greatly reduced and the equilibrium displaced to the right. The results so obtained, Table IB, are consistent with the assumption of equilibrium. High reactivations are obtained with very low reactivator concentrations. With TEPP even at these low concentrations it appears that the rate is much less than 1 minute and that the measurements constitute equilibrium values. The DFP data seem to indicate a rate, but the situation is not clear.

The quaternary oxime is a million times better than the non-methylated compounds and 50,000 times better than picolinohydroxamic acid in reactivating TEPP inhibited enzyme. The reactivation appears to be approaching enzyme speeds.

Table I.—Percent reactivation of alkylphosphate-inhibited acetylcholinesterase with 2-pyridine aldoxime methiodide

The reactivations were carried out as follows: 0.2 ml. of enzyme solution prepared from *Electrophorus electricus* was treated with 0.01 ml. of TEPP or DFP solution (20 γ /ml.), and diluted after 1 hour in the cold to 1.5 ml. This solution was used as stock for reactivation; to 0.2 ml. were added 0.2 ml. of reactivator solution of suitable concentration in 0.015 M phosphate buffer (pH 7) and 0.007 M EDTA. After suitable incubations (1', 5', 11') the reactivated solution was diluted to 50 ml. with water and 1 ml. was added to the manometric vessels for assay. The total enzyme dilution was 5625 fold. In part B the inhibited enzyme was diluted to 80.0 ml. instead of 1.5 ml.

A. DILUTED 7.5 TIMES

	TEPP		DFP	
	1'	5'	1'	5'
5.10 ⁻³ M			55	82
2.10 ⁻³			43	68
10 ⁻³	94	94		
10 ⁻⁴	41	48		
10 ⁻⁵	8	9.5		

B. DILUTED 400 TIMES

	1'	5'	1'	11'
10 ⁻⁴			38	53
10 ⁻³	85	89	9	12
10 ⁻⁶	25	29		

Most reactivators react directly with TEPP and DFP. This is also the case with the quaternary oxime. The rate as judged by acid production was fairly rapid but not extraordinary; with 0.01 M oxime and 0.002 M phosphate anhydride the time for 50% reaction at 25° C. and pH 7.4 was 12 and 20 minutes for TEPP and DFP respectively.

The nicotino- and picolinohydroxamic acids proved already to be of value as antidote of the so-called "nerve gas" in animals. The quaternary oxime is thousands of times more potent as a reactivator. It appeared desirable, therefore, to investigate whether this compound was sufficiently nontoxic to be used as an antidote and whether, if so, it would in fact serve as an antidote for the irreversible inhibitors of cholinesterase. White mice weighing about 20 g. were used without regard to sex. The general procedure was to inject paraoxon subcutaneously followed 1-2 min. later by interperitoneal injection of 2-pyridine aldoxime methiodide (2-PAM). In some cases multiple injections of 2-PAM were used starting 1-2 min. before the paraoxon, repeated 5 or 10 min. after the poison and in some cases again 15 min. later. The doses of paraoxon designated as LD₅₀ and LD₁₀₀ correspond to acute poisoning; death occurs in 5-20 min.

The acute toxicity of 2-PAM is given in Table II. The toxicity of the compound, while important, is not especially great. Death which is due to respiratory failure occurs within 10-20 min.

On the basis of the minimal lethal dose it was decided to use as a maximum dose 75 mg. 2-PAM/kg. This was considered to be a safe dose. The antidotal properties of 2-PAM as seen from Table III are quite marked. Complete survival was obtained from a LD₁₀₀ dose of paraoxon with a safe dose of 2-PAM. Because of uncertainty concerning the time course of an effective antidote level, it was thought that better results might be obtained with very low antidote doses if they were given as multiple injections. As will be seen, three injections totaling only 11 mg./kg. save 7 out of 11 mice.