DESIGNING OF A NEW DRUG WITH ANTIDOTAL PROPERTIES AGAINST THE NERVE GAS SARIN*

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Acetylcholinesterase and other hydrolytic enzymes are irreversibly inhibited by low concentrations of certain phosphorus-containing compounds, such as diisopropyl fluorophosphate (DFP), tetraethyl alkyl pyrophosphate (TEPP) and isopropoxymethyl fluorophosphonate (Sarin). Some of these compounds are chemical warfare agents (nerve gases) and others find use as insecticides. Their toxicity derives from the remarkable facility with which they inhibit acetylcholinesterase[1,2].

The inhibition is effected by a phosphorylation of the enzyme[3,4,5]. A basic group in the active site which in the normal catalytic process is acylated, is here phosphorylated[6,7]. The inhibited enzyme can be dephosphorylated and thus reactivated by nucleophilic agents[6,8]. By applying principles of molecular complementariness and physical organic chemistry to the design[9,10,11] of a reactivator, a very active *in vitro* reactivator, pyridine-2-aldoxime methiodide (2-PAM), was obtained[12].

In this compound the oxime functional group and the quaternary structure are so disposed that it is bound by the inhibited enzyme in a manner which directs the oxime function against the nucleophilic phosphorus atom. Shortly afterwards and independently this compound was also described by CHILDS, DAVIES, GREEN AND RUTLAND[13].

PAM is a very effective antidote for many of these poisons, especially when used with atropine[14–19]. The effectiveness of PAM as an antidote indicates that its *in vivo* penetration of tissues overlaps a very important part of the volume of tissue penetrated by the poisons. The very poor solubility of PAM in lipid solvents suggests that the penetration of PAM is restricted to the peripheral nervous system and this expectation is substantiated by the observation that PAM produces little reactivation of acetylcholinesterase in brain *in vivo*[20], although it produces very substantial reactivation of acetylcholinesterase in muscle[21].

PAM is effective none the less because the primary damage produced by these poisons is often in the peripheral nervous system. Since some alkyl-phosphates produce strong central effects, it seemed to us rather interesting to attempt to prepare a derivative of PAM which would have better possibilities of reaching the central nervous system and barring that, would at least have very different penetration

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characteristics. We therefore undertook the preparation of a "lipid-soluble PAM" which would yet be active, to be used in conjunction with PAM so that between them they penetrate a greater volume of the tissue reached by the poison. A feasible method of accomplishing this purpose is to replace the methyl group by a long chain–pyridine-2-aldoxime-dodeciodide (PAD).

It was found that PAD is much more soluble in chloroform than in water (Table I) and that it is about one third as active (on a molar basis) as PAM in reactivating inhibited acetylcholinesterase (Table II). Sarin was chosen as the poison to use since it appears that this compound produces rather more central effects* than some of the other poisons of this very broad class of compounds. The other facet of activity which may contribute toward antidotal properties in some cases is the direct reaction between the antidote and the poison[23]. PAD reacts with Sarin at about the same rate as PAM. Thus both aspects of activity are retained.

TABLE I

SOLUBILITY OF PAM AND PAD

	H_2O	$CHCl_3$
PAM	$2.7 \cdot 10^{-1}$	$3 \cdot 10^{-6}$
PAD	$1.3 \cdot 10^{-3}$	$1.2 \cdot 10^{-1}$

Molar solubilities of PAM and PAD at room temperature in water and chloroform. These values are approximate.

TABLE II

ACTIVITY OF PAM AND PAD

	k' (min^{-1})	$t_{1/2}$ (min)
PAM	0.30	2.3
PAD	0.11	6.2

Comparison of PAM and PAD as reactivators for TEPP inhibited acetylcholinesterase from electric eel. The concentrations of reactivators were $5 \cdot 10^{-6} M$ in 0.03 M NaCl, $10^{-3} M$ phosphate buffer at pH 7.9, $10^{-4} M$ EDTA at 25°. The constant k' is the pseudo first order specific rate constant $k' = -2{,}3/t \log$ (fraction still inhibited).

The time required for 50% reactivation, $t_{1/2}$, is given as an aid in appreciating the activity of these compounds.

The results of antidote experiments with mice are shown in Table III. The amount of PAD, 15 mg/kg is equivalent to 3 mg/kg of PAM in *in vitro* reactivating power (as measured at a concentration of $5 \cdot 10^{-6} M$). Therefore when PAD was used, the amount of PAM was decreased to maintain a constant reactivating potential. It is quite clear that the addition of PAD leads to a very greatly increased survival rate, *i.e.* complete survival is obtained under conditions where in its absence almost complete mortality occurs. PAM alone increases the LD_{50} by a factor of about 1.5**, PAM combined with atropine by a factor of about 2, and finally with the addition of PAD the LD_{50} is increased roughly 3.5 fold.

* Central and peripheral effects of these poisons are discussed by HOLMES[22].

** It should be noted that because of the sharpness of the mortality dose curve an increase of LD_{50} by what might appear to be a small amount (a factor of 1.5) is sufficient to convert complete mortality to complete survival.

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TABLE III

ANTIDOTAL PROPERTIES OF PAD

(with PAM and atropine)

Atropine mg/kg	*PAM mg/kg*	*PAD mg/kg*	*Sarin mg/kg*	*Multiple LD_{50}*	*No. of animals*	*Survivors*
10	80	0	0.8	2.7	10	2
10	77	15	0.8	2.7	10	10
10	0	15	1.0	3.4	10	0
10	80	0	1.0	3.4	10	0
10	77	15	1.0	3.4	15	8

$LD_{100}/LD_{50} = 1.16$

Atropine was given 30 minutes before Sarin and PAM and PAD 10 minutes before the poison.

We have by a very simple modification altered the *in vivo* permeability characteristics of a drug. This principle of modification would appear to have rather wide possibilities of application.

METHODS

Pyridine-2-aldoxime dodec-iodide was prepared by heating at about 100°, 12.2 g of pyridine-2-aldoxime[24] with 29.6 g of dodecyliodide in 100 ml of dimethylformamide for 10 h. The solvent was removed under reduced pressure and the remaining dark green oil was dissolved in 100 ml of hot 20% methanol and allowed to cool. The mixture was shaken with three volumes of ether. Three layers formed. The bottom and top layers were discarded. The middle layer, a thick oil, was treated again with fresh ether and yellow crystals of product separated. Recrystallized from 20% methanol; yellow plates, m.p. 159°–160° C, yield 15%; analysis–neutral equivalents; found 419 (potentiometric titration in 10% aqueous ethanol), theory 418.

Solubilities were estimated at room temperature by preparing saturated solutions with slightly excess solid. The solution was filtered and diluted if necessary so that an absorption spectrum could be obtained (Cary recording spectrophotometer) and compared with that of a known concentration.

In the animal work, white mice weighing 20–30 g were used without regard to sex. Sarin* was injected subcutaneously and atropine, PAM and PAD intraperitoneally. The compounds were dissolved in 0.9% NaCl solution save PAD which because of its poor water solubility was administered in propylene glycol. Not more than 1% of the body weight was introduced in any one injection.

The enzyme used in the reactivation experiments was purified acetylcholinesterase from the electric organ of *Electrophorus electricus*.

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SUMMARY

Pyridine-2-aldoxime dodec-iodide (PAD) was synthesized on the expectation that it would be much more lipid-soluble than the methiodide (PAM) and would therefore penetrate, *in vivo*, tissues which were not permeable to PAM. If such were the case it was anticipated that PAD might augment the antidotal properties of PAM in those instances of alkylphosphate intoxication in which the significant area of penetration of the poison did not lie wholly within the sphere of penetration of PAM. While PAM is readily soluble in water and very poorly soluble in chloroform, PAD is just the reverse—readily soluble in chloroform and poorly soluble in water. PAD is about one third as active as PAM as an *in vitro* reactivator of tetraethylpyrophosphate inhibited acetylcholinesterase when compared at $5 \cdot 10^{-6} M$ concentration.

* Sarin was obtained from Drs. JANDORF AND SUMMERSON of the U.S. Army Chemical Center, Edgewood, Md.

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With white mice poisoned with Sarin (isopropoxymethyl fluorophosphonate) PAD quite remarkably extends the antidotal properties of PAM + atropine.

This very simple principle of replacing a short chain by a very long chain has served to alter the *in vivo* penetration of a drug and extend its application.

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Short Communications

Test for isocitritase and malate synthetase in animal tissues

The ready incorporation of labelled carbon atoms of fatty acids into carbohydrates which can occur in animal tissues does not necessarily represent a net conversion of fat to carbohydrates, and the question whether such a conversion occurs in higher animals is still open[1]. Such a conversion might be expected to occur under conditions where fatty acids are the chief source of energy, *e.g.* in rats maintained on a high fat diet, or in hibernating mammals, or in the chick embryo, the chief energy source of which is the fat store of the yolk[2].

A material in which a net synthesis of carbohydrate from fatty acids is known to occur is the germinating castor bean. Experiments on seedlings of this plant are in agreement with the view that the glyoxylate cycle[3,4] is a key step in this overall reaction in this material[5,6]. The experiments reported in the present paper were designed to assay the chief enzymes of the glyoxylate cycle —isocitritase[7] and malate synthetase[8]—in some animal tissues in which a net conversion of fat to carbohydrates has been suspected to occur. Tissues of rats maintained on high fat diets and chick embryos were used. The assay methods were the same as those used in previous experiments from this laboratory on extracts of *Pseudomonas* KB1[4].

Adult female rats (4–5 months old, weighing 190 g) were placed on two types of high-fat diet. Diet I contained 10% casein, 2% cod liver oil, 2.5% McCollum's salt mixture[9] (modified to reduce the sodium content), 0.1% choline, B-group vitamins: thiamine HCl, pyridoxine and nicotinamide (10 mg each/kg diet), Ca pantothenate and riboflavine (20 mg each/kg diet) and 85.4% butter. Diet II was the same as I except that no protein was present and the butter content was 95.4%. The rats on Diet I lost weight for the first week, then regained weight and levelled off at 200 g; their condition was good. The rats on Diet II lost weight steadily, decreasing on average from 192 g to