Antagonism of Phospholine (Echothiophate) Iodide* by Certain Quaternary Oximes. (25904)

ROBERT A. LEHMAN AND MAUDE E. NICHOLLS
Research Labs., Campbell Pharmaceuticals, N. Y. City

It was previously reported that 2 oximes bearing quaternary nitrogen atoms were highly effective against Phospholine Iodide toxicity when both were injected intraperitoneally in the mouse(1). These compounds were Protopam Iodide (pralidoxime iodide, 2-PAM, 2-pyridine aldoxime methiodide) and TMB4 (1, 1'-trimethylene-bis(4-formylpyridinium bromide)dioxime). Phospholine is under investigation in treatment of myasthenia gravis(2) and systemic actions have been observed during its use as a topical miotic in glaucoma(3). Hence it appeared important to investigate its relationship to the oximes further and to compare antidotal activity of the oximes by oral and parenteral administration. TMB4 and Protopam are, of course, also of interest in connection with poisoning due to anticholinesterases which may be used as pesticides or chemical warfare agents(4,5).

Methods. Acute lethal doses were estimated in white male mice of CF1 strain and 25 ± 5 g in weight. Concentrations were such that total solution administered fell between 0.1 and 1.5 cc. Doses were spaced in most cases by a factor of 1.41 with 5 animals at each dose level. Deaths were counted at the end of 24 hours but actually 92% of all deaths occurred within one hour and 83% within 15 minutes. Symptoms came on within a few minutes and had entirely disappeared in animals surviving 24 hours. The LD_{50} and its standard error was calculated by the method of Miller and Tainter(6). The experimental design consisted in estimation of the LD_{50} of Phospholine with and without administration of a selected dose of antidote. Phospholine was always given intraperitoneally. Intraperitoneal injection of an oxime was given within a few seconds of Phospholine. Oral administration of an oxime was by stomach tube at intervals of 30 minutes to 24 hours before Phospholine. TMB4 was synthesized by the method of Poziomek and coworkers(7) and appeared to be identical with a sample kindly supplied by Dr. B. J. Jandorf of the U.S. Army Chemical Warfare Labs. Protopam Iodide was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. and recrystallized from aqueous isopropanol: m.p. 220—2°C decomp.; E_M at 294 mmu, 12,500. Protopam Chloride was prepared by passing a saturated solution of Protopam Iodide through a column of anion exchange resin, evaporating the effluent and recrystallizing from a 1:1 mixture of absolute methanol and ethanol; m.p. 229—30°C decomp.; E_M at 294 mmu, 12,600; analysis, theory N-16.23%, Cl-20.53%, found N-15.94%, Cl-20.40%.

Results. The LD_{50} of Phospholine after oral or intraperitoneal administration of oximes is given in Table I. Slopes for dosage mortality curves in units of logarithms and probits are given for each LD_{50}. The control LD_{50} for Phospholine Iodide without oximes was taken as 0.229 ± 0.0057 which is a weighted mean of 9 determinations. Range was from 0.200 ± 0.013 to 0.256 ± 0.019. Slopes ranged from 8.5 to 16.1 with a mean of 12.0.

Lethal doses of Protopam Iodide and Chloride were respectively 235 ± 32 mg/kg (.91 ± .12 millimol/kg) and 205 ± 14 mg/kg (1.19, ± .081 millimols/kg) by intraperitoneal injection and ca. 4000 mg/kg (15.2 millimols/kg) and 4100 ± 890 mg/kg (23.7 ± 5.1 millimols/kg) by oral administration. The oral LD_{50} of Protopam Iodide could only be approximated because of poor water solubility. As antidotes, the 2 compounds administered intraperitoneally at .58 millimol/kg raised the LD_{50} of Phospholine to 12.6 ± 1.4 for the Chloride and 12.8 ± 2.1 for the Iodide. In the same way the LD_{50} of Phospho- 60 minutes after an oral dose of 4.6 millimols/kg of the Chloride was 5.4 ± .86

* Diethoxyphosphinylthiocholine iodide.

TABLE I. Effect of Oximes on Lethal Dose of Phospholine Iodide in the Mouse.

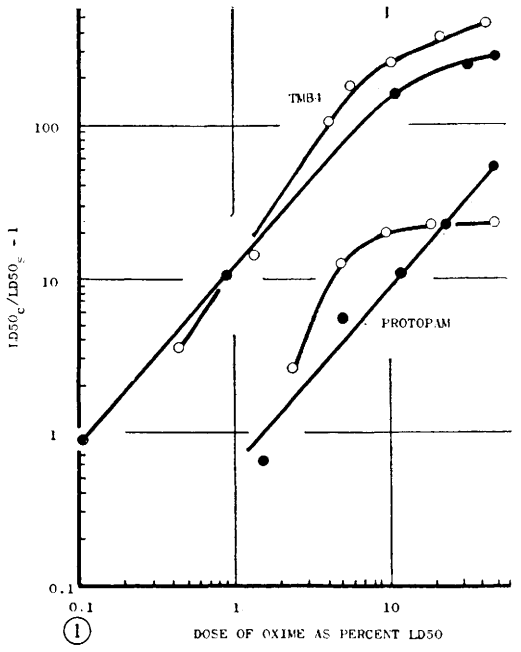
Oxime and route of admin.	Dose of oxime in mg/kg	Time between admin. of oxime and Phospholine	LD_{50} of Phospholine ± stand. error	Slope*
TMB4, intraper.	.1	0	.43 ± .057	11.2
	1		2.70 ± .38	7.5
	12		36 ± 4.9	7.7
	36		55 ±11	5.5
	54		64 ± 4.7	14.1
" , oral	20	30 min.	1.06 ± .10	11.2
	62.5		3.5 ± .45	8.1
	200		23.6 ± 2.2	11.2
	250		39.5 ± 6.2	5.5
	500		58 ± 9.7	5.2
	1000		85 ±12	7.5
	2000		103 ±14	7.6
	20	60 min.	.74 ± .12	6.7
	2000		127 ±20	5.5
	20	120 min.	.92 ± .11	8.7
	2000		135 ±17	8.2
	20	240 min.	.46 ± .060	11.2
	2000		90 ±14	5.4
	20	24 hr	.217± .025	12.7
	2000		1.64 ± .23	7.4
Protopam chloride, intraper.	3	0	.38 ± .026	16.1
	10		1.47 ± .19	11.8
	25		2.70 ± .20	27.0
	50		5.3 ± .83	5.5
	100		12.6 ± 1.4	7.7
" " , oral	100	30 min.	1.00 ± .14	10.5
	800		3.2 ± .57	8.3
	100	60 min.	.84 ± .11	10.9
	200		3.1 ± .81	4.1
	400		4.8 ± .75	5.6
	800		5.4 ± .86	5.4
	2000		5.6 ± .38	15.4
	100	120 min.	.46 ± .063	10.6
	800		4.1 ± .63	9.6
	100	240 min.	.33 ± .055	6.3
	800		2.6 ± .32	8.3
	100	24 hr	.205± .033	6.6
	800		.221± .031	7.5

* Slope estimated graphically and expressed in units of log dose and probit mortality.

and of the Iodide 7.7 ± 1.1. It is felt that these 2 salts are pharmacologically equivalent. Therefore, all subsequent work was carried out with the Chloride since this would appear to be the preferable salt for clinical use due to its excellent solubility and avoidance of possible side reactions to iodide ion.

In Fig. 1 data for the effect of TMB4 and Protopam Chloride by intraperitoneal injection and for the same drugs 30 and 60 minutes respectively after oral administration on LD_{50} of Phospholine have been transformed and plotted on logarithmic scales. Doses of oxime expressed as percentage of lethal dose appear as abscissae and $\frac{LD_{50_C}}{LD_{50_S}} - 1$ as ordinates,† where LD_{50_C} and LD_{50_S} refer to LD_{50} of Phospholine with and without antidote respectively. As a practical matter, doses of antidotes were not carried beyond one-half of their LD_{50} at which level mortality did not occur from the oxime alone.

† (x-1) of Schild(8).



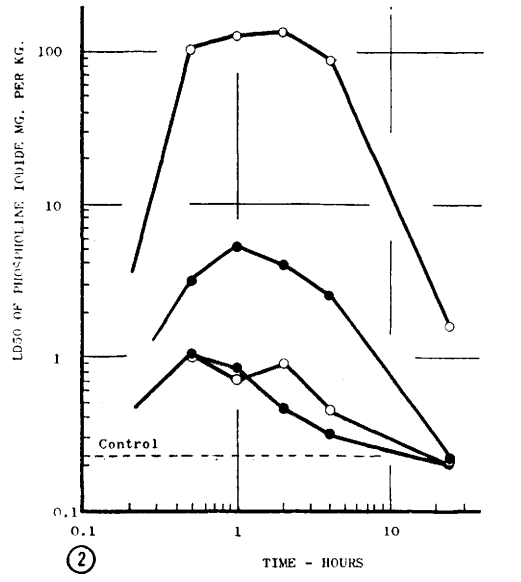


FIG. 1. Relationship of log dose of oxime expressed as % LD_{50} and log $\left(\frac{LD_{50_C}}{LD_{50_S}} - 1\right)$ where LD_{50_C} and LD_{50_S} refer to lethal dose of Phospholine with and without oxime respectively. ○, oral administration of TMB4, 30 min., and Protopam, 60 min., before Phospholine. ●, intraper. inj. of TMB4 and Protopam.

FIG. 2. Relationship of LD_{50} of Phospholine to time interval between oral administration of oxime and estimation of LD_{50}. ○, TMB4 at 2000 mg/kg (upper graph) and 20 mg/kg (lower graph). ●, Protopam at 800 mg/kg (upper graph) and 100 mg/kg (lower graph).

TABLE II. Dose of Oximes Necessary to Raise the LD_{50} of Phospholine by a Factor of 10.

	Dose of oxime mg/kg	Dose of oxime % of LD_{50}	Therapeutic index*
Protopam chloride			
Oral	176	4.3	23
Intraper.	24	11.5	9
TMB4			
Oral	40	.88	114
Intraper.	.86	.83	120

* LD_{50}/ED_{50} where effective dose is that necessary to raise LD_{50} of Phospholine 10-fold.

The curves for TMB4 are linear over a considerable range of doses after which curvature appears. Oral and intraperitoneal plots are remarkably similar in shape and position so long as dose of oxime is expressed in terms of LD_{50}. Hence the relative activity of this drug by the 2 routes is approximately equal to the inverse ratio of its oral to intraperitoneal LD_{50} or 1 to 43. At 50% of LD_{50} of TMB4 the LD_{50} of Phospholine was raised by factors of 450 and 280 for oral and intraperitoneal administration respectively.

In the case of Protopam oral and intraperitoneal curves are not as much alike, the former showing a definite ceiling while the latter gives no sign of curvature up to one-half LD_{50}. Furthermore, the range of doses over which Protopam is effective is narrower than in the case of TMB4. At 50% of the LD_{50} of Protopam the LD_{50} of Phospholine was raised by factors of 23 and 50 for oral and intraperitoneal administration respectively.

Comparison of activities of the oximes in terms of percentages of LD_{50} which cause a 10-fold increase in the LD_{50} of Phospholine (graphical interpolation at 9 on the axis of ordinates of Fig. 1) is given in Table II. The reciprocals of these percentages multiplied by 100 are therapeutic indices at the arbitrarily selected activity level and are also given in the Table. Protopam has a better therapeutic index orally than intraperitoneally, while TMB4 is the same by either route. TMB4 has an index 5 times as great as Protopam by oral administration and 13 times by intraperitoneal injection.

Oral activities of the oximes were determined by injection of Phospholine after various intervals and in Fig. 2 is shown the relationship of time interval to LD_{50} of Phospholine. Doses of oximes were selected from the results in Fig. 1 as those that would be expected to show roughly maximum and minimum effects. Peak responses are not sharp and are spread between 30 and 120 minutes depending on dose level. Except for TMB4 at a high dose level, the effect of the oximes was completely dissipated after 24 hours. In comparing the data obtained by oral administration (Fig. 1), it should be understood that TMB4 was tested at a 30 minute interval while Protopam was at 60 minutes. In view of the results in Fig. 2 it is not felt that this discrepancy has any practical significance. The origin for the curves in Fig. 2 is uncertain. There would probably be some protective effect even if both Phospholine and oxime were given simultaneously since a certain amount of absorption of oxime would occur from the intestinal tract during the few minutes required for absorption of Phospholine from the peritoneal cavity.

Discussion. An extensive literature has developed on use of Protopam and TMB4 against poisoning by Sarin, TEPP, Parathion, isoflurophate and others. Here, the antidotal action of the oximes is of a limited order of magnitude unless atropine is also given. This is generally assumed to be due to the failure of the quaternary oximes to enter the central nervous system in contradistinction to the above group of cholinesterase inhibitors which do so. Phospholine appears to be the only phosphate type anticholinesterase bearing a quaternary nitrogen atom which is presently in clinical or industrial use and the marked effectiveness of the oximes without atropine presumably depends upon the fact that Phospholine also fails to cross the blood brain barrier.

Both oximes have potential value as antagonists to Phospholine and Protopam is already being used for this purpose. While TMB4 has the greater therapeutic index the activity of Protopam nevertheless would appear to be more than adequate for practical purposes.

It is of interest to consider the nature of the antagonism of Phospholine by quaternary oximes. The slopes in Table I show considerable variation which appears to be random with no systematic progression throughout any series. As would be expected, slopes for replicate assays of Phospholine alone are more consistent. While a rigorous statistical test does not seem justified because of the small number of animals used for each LD_{50}, there is probably no significant departure from parallelism among dosage mortality curves for Phospholine alone and when given with antagonists. This observation, together with the linearity over a considerable range of the curves relating $\log\left(\frac{LD_{50_C}}{LD_{50_S}}-1\right)$ and log dose of oxime (in units of LD_{50}), furnishes presumptive evidence of competitive antagonism(8). However, such a conclusion is perhaps hazardous when dealing with a system as complex as the whole animal and in view of the fact that tissue concentrations of the oximes may not be linearly related to dose. *In vitro* experiments have shown that both oximes reactivate the acetylcholinesterase of whole mouse blood which has been exposed to Phospholine and it has been assumed that this is the basis of the antidotal action *in vivo* (1). However, degree of reactivation of the enzyme possible in a given time falls slowly as the exposure period over which Phospholine is allowed to react increases (24% in 2 hours). Since the mice used in the present experiments died very promptly after a lethal dose was administered, it is reasonable to ignore this factor. It is sugestged that the antagonism of Phospholine by the oximes is competitive within the first hour or 2 during which Phospholine is probably acting as a whole molecule, and that a different relationship must obtain in later periods. It is not yet clear whether this is due to phosphorylation of the enzyme accompanied by splitting of the Phospholine molecule or to conversion of an enzyme—diethyl phosphate to monoethyl phosphate as suggested by the work of Cohen, *et al.*(9).

Summary. Protopam (pralidoxime) Chlor-

ide (or Iodide) and TMB4 (1, 1'-trimethylene-bis(4-formylpyridinium bromide) dioxime) when injected intraperitoneally in the mouse at 50% of LD_{50}, raise the intraperitoneal lethal dose of anticholinesterase, Phospholine (echothiophate) Iodide, by factors of 50 and 280 respectively. When the same oximes are given orally 30 to 60 minutes before Phospholine the intraperitoneal LD_{50} is increased by factors of 23 and 450. Graphs relating log dose of oxime and log dosage ratio of Phospholine are linear over a considerable range. The therapeutic indices for arbitrary increase of 10 times in LD_{50} of Phospholine are 23 and 9 for Protopam and 114 and 120 for TMB4 by oral and intraperitoneal administration respectively. The possible nature of the antagonism is discussed.

1. Lehman, R. A., Fitch, H. M., Bloch, L. P., Jewell, H. A., Nichells, M. E., *J. Pharmacol. Exp. Therap.*, 1960, v128, 307.

2. Osserman, K. E., Cohen, E. S., Genkins, G., *Proc. 2nd Internat. Myasthenia Gravis Symp.*, Los Angeles, April 19, 1959.

3. Drance, S. M., Carr, F., *Am. J. Ophthalmol.*, 1960, v49, 470.

4. Namba, T., Hiraki, K., *J. Am. Med. Assn.*, 1958, v166, 1834.

5. Wills, J. H., Kunkel, A. M., Brown, R. V., Groblewski, G. E., *Science*, 1957, v125, 743.

6. Miller, L. C., Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 261.

7. Poziomek, E. J., Hackley, B. E., Steinberg, G. M., *J Org. Chem.*, 1958, v23, 714.

8. Schild, H. O., *Pharmacol. Rev.*, 1957, v9, 242.

9. Cohen, J. A., Oosterbaan, R. A., Jansz, H. S., Berends, F., *J. Cell. Comp. Physiol.*, 1959, v54, 231.

Received May 12, 1960. P.S.E.B.M., 1960, v104.

Comparative Inhibition of Pyridoxal Kinase and Glutamic Acid Decarboxylase by Carbonyl Reagents.* (25905)

DONALD B. MCCORMICK,† BEVERLY M. GUIRARD AND ESMOND E. SNELL
Dept. of Biochemistry, University of California, Berkeley

γ-Aminobutyric acid (γABA) has been implicated as a transmission regulator in the central nervous system(1), though experimental evidence for such a role is ambiguous. Formation of this compound in brain from L-glutamic acid is catalyzed by a decarboxylase which requires pyridoxal phosphate as coenzyme(2). Carbonyl reagents are known to inhibit most pyridoxal phosphate enzymes through formation of a coenzymatically inactive derivative with the cofactor(3). Many of these reagents are now recognized as stimulants of the central nervous system ("psychic energizers") which in sufficient excess, produce epileptiform seizures that may be alleviated by administration of Vit. B_6(4). Localization of glutamic decarboxylase in brain(2), together with an observed reduction in levels of γABA following treatment by carbonyl reagents(5) have been interpreted to mean that both analeptic and convulsive activities of active carbonyl reagents may result from interference with production of this inhibitor of neuronal transmission by the decarboxylase (6). However, pyridoxal kinase catalyzes formation of pyridoxal phosphate from Vit. B_6 and has been shown to be extremely sensitive to inhibition by carbonyl reagents(7). The possibility therefore exists that the decrease in concentration of γABA results from lowered pyridoxal phosphate levels as a consequence of inhibition of pyridoxal kinase rather than from inhibition of the glutamic decarboxylase *per se*. This report demonstrates that pyridoxal kinase is much more sensitive than glutamic decarboxylase to inhibition by carbonyl reagents both *in vitro* and *in vivo*. These findings emphasize the possibility that inhibition of the kinase, with consequent decrease in pyridoxal phosphate production,

* Supported in part by grant from Nat. Inst. Health, U. S. P. H. S.

† Present address: Graduate School of Nutrition, Cornell Univ., Ithaca, N. Y.