Chemotherapeutic Effectiveness of 1,1'-Trimethylene Bis (4-Formylpyridinium Bromide) Dioxime (TMB-4) in Experimental Anticholinesterase Poisoning. (23955)

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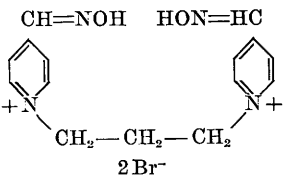
Treatment of serious poisoning by phosphate and phosphonate esters of the "nerve gas" and insecticide type relies principally on prevention and control of the actions of acetylcholine(1,2,3). Long-lasting "irreversible" inhibition of body cholinesterase by these esters in small amounts is believed to account for their great toxicity(4,5) and control of their actions has centered on liberal use of atropine, long known for its capacity to block the actions of acetylcholine in many of its loci of action, including the central nervous system(1,2,6,7). Although the symptom-relieving and life-saving capacity of atropine is considerable when given in large amounts early in poisoning coupled with artificial respiration(8), its use is nevertheless limited. No other compounds with atropine-like properties, offering substantial improvement, have been found(8).

Repair of the biochemical lesion produced by nerve gas and similarly-acting phosphate esters (*e.g.* certain insecticides) has been a more recent approach to the problem(5). Restoration of cholinesterase activity to physiologically adequate levels with sufficient rapidity to avert or halt progress of poisoning serious enough to be beyond the control of atropine has been the object of this approach. The approach attempts to "cure the disease" by restoring the body mechanisms for dealing with its acetylcholine competently, rather than by treating symptomatically with a drug such as atropine. Suggestions that such an approach might be feasible appeared when it was demonstrated that certain oximes and hydroxamic acids may catalyze breakdown of such phosphate esters to innocuous products, may restore cholinesterase activity, or perform both functions *in vitro*(5,10). Limited *in vivo* data appear to demonstrate chemotherapeutic effectiveness of 2-pyridine aldoxime methiodide (2-PAM) and other oximes against some phosphate esters(10,11). However, other investigators pointed out that in the absence of atropine the effectiveness of 2-PAM was negligible against phosphate esters(12). The present preliminary report deals with the effectiveness of a quaternized bispyridine aldoxime, one of a series emerging from a systematic synthesis of related compounds(13). This compound is 1,1'-Trimethylene bis (4-formylpyridinium bromide) dioxime (hereafter designated TMB-4) represented by the structural formula:



It reactivates GB-inhibited cholinesterases *in vitro* at a higher rate than does 2-PAM(13).

Methods. Briefly, the method for assessing chemotherapeutic effectiveness is as follows. Tests were conducted in 5 different species. A minimal number of animals was used in this routine screening method for preliminary evaluation of candidate agents. The toxic phosphate ester against which TMB-4 was tested was isopropyl methylphosphonofluoridate (GB). Two general types of tests were conducted: a) "prophylactic," in which TMB-4 was given not earlier than 2 minutes before GB to minimize excretion or other disposition of the compound; and b) "therapeutic," in which GB was followed not later than 30 seconds by TMB-4. Both compounds were in most cases given intravenously to avoid absorption problems; exceptions will be noted later. 2-PAM and TMB-4 were given routinely in maximum symptom-free doses, except in rabbits where equal amounts were used. GB was injected in appropriate multiples of its LD_{50}'s. Since it became apparent

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TABLE I. Protective Action of 2-Pyridine Aldoxime Methiodide (2-PAM) and of 1,1′ Trimethylene Bis (4-Formylpyridinium Bromide) Dioxime (TMB-4) against Experimental "GB" Poisoning in Different Species.* *Poisoning dose of GB used:* Mice—.173 mg/kg (LD_{99}); rats—.126 mg/kg (2 LD_{50}); rabbits—intrav. .340 mg/kg (20 LD_{50}), subcut. .700 mg/kg (20 LD_{50}); dogs and cats—intrav. .440 mg/kg (20 LD_{50}), subcut. .900 mg/kg (20 LD_{50}).

	2-PAM				TMB-4			
		Survival ratio "Prophylactic"		"Therapeutic"		Survival ratio "Prophylactic"		"Therapeutic"
	Dose, mg/kg	Alone	With atropine	Alone	With atropine	Dose, mg/kg	Alone	With atropine	Alone	With atropine
Mice	40	0/10		0/10		12	0/10		1/10	
Rats	40	0/6		0/6	1/6	25	0/6		1/6	6/6
Rabbits	5		0/5		2/6	5		2/5		4/6
Cats	40		2/5		5/5	20		5/5		3/5
Dogs	40		5/5		4/5	20		5/5		4/5

* For description of test, see text.

from its structure that TMB-4, like 2-PAM has little protective action in the absence of atropine(7), the latter was included, as indicated, in standard dose of 4 mg/kg in rats, 2 mg/kg in rabbits, and 0.5 mg/kg in cats and dogs. Use of the terms "prophylactic" and "therapeutic" is not intended to place interpretation on mechanism of action, but rather refers to temporal relation of administration of toxic and treatment agents to test animals. Under conditions of our tests, 2-pyridine aldoxime (2-PAM) exerts insignificant prophylactic and therapeutic action in mice and rats. Atropine, which is known to be relatively ineffective against anticholinesterase poisoning in rodents, improved both actions of 2-PAM somewhat; the most striking contribution of atropine is seen in cats and dogs. Whereas rabbits, cats, and dogs die of intravenous lethal dose of GB within a few minutes, a combination of 2-PAM and atropine protects these animals considerably against 20 LD_{50}'s of GB prophylactically and therapeutically in doses which are ineffective by themselves. In rats TMB-4 is more effective than 2-PAM. However, recovery time from symptoms of

TABLE II. Recovery Periods of Animals Poisoned with GB and Treated with 2-PAM or TMB-4 as Adjuncts to Atropine.

	2-PAM				TMB-4			
	"Prophylactic"		"Therapeutic"		"Prophylactic"		"Therapeutic"	
Species	No. of animals tested	Remarks	No. of animals tested	Remarks	No. of animals tested	Remarks	No. of animals tested	Remarks
Rats			6	5 died within 10 min. 1 recov. within 60 min.			6	Recov. within 15 min.
Rabbits	5	Died within 3 min.	6	4 died within 10 min. 2 recov. within 3 hr	5	3 died within 10 min. 2 recov. within 30 min.	6	2 died within 20 min. 4 recov. within 2 hr
Cats	5	3 died within 15 min. 2 recov. within 5 hr	5	Recov. within 5 hr	5	Recov. within 5 hr	5	2 died within 24 hr 3 did not recover within 24 hr
Dogs	5	Did not recov. within 24 hr	5	1 died within 60 min. 4 did not recover, 24 hr	5	Recov. within 1½ hr	5	1 died within 60 min. 4 recov. within 3 hr

poisoning was considerably shorter in all species except in cats when TMB-4 was administered with atropine and used as "therapeutic" agent. It is to be noted that in these "therapeutic" tests, GB was given subcutaneously only because death from 20 intravenous LD_{50}'s of GB is so rapid that timely administration of treatment agent is practically impossible. The intravenous treatment was started as soon as poisoning symptoms were visible. Effectiveness of TMB-4 in the different species may be compared with that of 2-PAM by reference to Tables I and II.

Discussion. Conditions of "prophylactic" and "therapeutic" tests allow minimal protective action to appear, while at the same time requiring antidotal action significantly above that of atropine to be detected. The intravenous route of administration avoids most difficulties in interpretation arising from changes in rate and extent of absorption of toxic and treatment compounds. The use of atropine recognizes the necessity for blocking immediate and very rapid lethal action of GB, by blocking of muscarinic and central nervous system action of acetylcholine(7). This provides more time than otherwise possible for the action of cholinesterase restoratives such as TMB-4, as revealed by *in vitro* studies(13), presumably to bring tissue cholinesterase to lifesaving or even physiologically adequate levels. The latter deserves emphasis, for survivors among cats and dogs exposed to sublethal doses of GB and treated with atropine alone show weakness, gait abnormalities and other signs of incomplete recovery for many hours or days, whereas in rabbits, cats and dogs fast recovery was found when treated with TMB-4 and atropine prophylactically. Similarly rats, rabbits and dogs exposed to multiple LD_{50}'s of GB show practically complete recovery in a few hours when treated also with TMB-4. Further study is obviously required to correlate rates of tissue cholinesterase restoration with return of function to levels required to ensure survival and normal activity. In most of these tests a lower dose of TMB-4 was administered than of 2-PAM for the reason given above; however, this does not necessarily indicate a lower therapeutic index of the latter compound. In this preliminary study the number of animals is too small for statistical evaluation of the superiority of one compound above the other. However, comparison of effectiveness of these compounds suggests that TMB-4 is at least equivalent if not superior to 2-PAM in its use as therapeutic agent in experimental anticholinesterase poisoning.

The quaternized bispyridine aldoxime with a trimethylene bridge between pyridine aldoxime portions of the molecule, TMB-4, is the most effective against experimental nerve gas poisoning and least toxic of the group. With increasing chain length the toxicity rises and therapeutic effectiveness diminishes.

The compound, 1, 1′-trimethylene bis (4-formylpyridinium bromide) dioxime (TMB-4), reported here seems very promising by comparison with 2-pyridine aldoxime methiodide (2-PAM), since when combined with atropine its effectiveness in immediate protection against lethal action and especially in symptomatic recovery from poisoning by GB is such as to merit trial against poisoning by other anticholinesterase agents such as parathion, tetraethylpyrophosphate, and diisopropyl phosphorofluoridate.

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Kinetics of Tyrosine Oxidation by Crude Tyrosinase Preparations from *Neurospora crassa*.* (23956)

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The ascomycete *Neurospora crassa*, strain 15300, is a mutant which differs from the wild-type at the albino-2 locus(1). Extracts of 15300 which contain the enzyme tyrosinase, are capable of oxidizing tyrosine, dopa (3-4 dihydroxyphenylalanine), or other mono- or dihydroxyphenols to corresponding 3-4 quinones, which are eventually converted to colored products (dopachrome in the case of tyrosine and dopa). We have for some time been engaged in an analysis of genetic control of synthesis and specificity of tyrosinase in strain 15300(2.3.4). The necessity to examine a large number of genetically different cultures has dictated the use of crude enzyme preparations and spectophotometric methods. This paper presents a study justifying the use of such methods and the use of crude extracts as a source of enzyme for determination of enzyme activity and specificity as reliable and dependable and not different from that of purified enzyme preparations.

Materials and methods. Large quantities of mycelium, from a single ascospore culture (15300-131a) isolated from the cross of 15300 A x 15300a, were obtained by growth on liquid minimal medium for 33 days at 25°C. After harvest, the mycelium was washed thoroughly and lyophilized. Enzyme preparations were made by homogenizing 23 mg of lyophilized mycelium in 2.50 ml of buffer (0.85% NaCl + 0.1 M phosphate pH 6.7) at 5°C for 5 minutes in an all-glass homogenizer. Aliquots of the supernate, collected after centrifugation at 5000 rpm for 20 minutes at 5°C, were tested for activity by measuring the rate of dopachrome formation spectrophotometrically, *i.e.* at 305 mμ or 475 mμ in a Beckman DU spectrophotometer. Reaction mixtures consisted of 0.25 ml of supernate + 3.25 ml of buffer containing defined amounts of l-tyrosine as substrate. Further details are provided elsewhere(4).

Results. Kinetics of dopachrome formation. The course of dopachrome formation in our reaction mixtures follows closely the course of tyrosine oxidation demonstrated by other workers using manometric methods and purified enzyme preparations (Fig. 1). There is a distinct induction period, followed by period of linearity, ultimately falling off as the reaction proceeds. Furthermore, the rate of dopachrome formation during the linear period is related to substrate concentration as expected on the basis of the Lineweaver-Burk modification of the Michaelis-Menten relationship (Fig. 2). This makes it possible to calculate both V_{max}, the reaction rate expected when substrate is unlimited, which measures enzyme concentration under these circumstances, and the apparent Michaelis Constant (K_s) of the enzyme. The rate of dopachrome formation is influenced by pH (Fig. 3), falling symmetrically on either side of pH 7.4, which can be taken as the pH optimum of the enzyme. All these observations strongly suggest that the spectrophotometric method is satisfactory for the measurement of tyrosine oxidation by crude tyrosinase preparations from *Neurospora*. A direct demonstration was nevertheless deemed important, and studies of the relationship of tyrosine utilization to dopachrome formation in our reaction mixtures were therefore undertaken.

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