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Bicyclic Phosphorus Esters: High Toxicity without Cholinesterase Inhibition

Abstract. *4-Isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-oxide has a mouse intraperitoneal lethal dose, 50 percent effective, of 0.18 milligram per kilogram of body weight. Related compounds used by many chemical researchers are also highly toxic. Brain acetylcholinesterase inhibition is not involved in their mode of action. The structural similarity of these compounds to adenosine 3',5'-monophosphate (cyclic AMP) is of interest.*

4-Alkyl derivatives of 2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane are extensively used for spectroscopic studies and are candidates for use as flame retardants, vinyl resin stabilizers, and antioxidants (*1*). The commercially available (*2*) 4-ethyl phosphite derivative forms a relatively stable ozone adduct useful as a convenient singlet oxygen source (*3*). Thus many investigators have studied and continue to study the chemistry of these compounds without an awareness of any unusual hazard. Our findings indicate that these bicyclic phosphorus compounds should be handled with great care because of their high toxicity to mammals.

The bicyclic compounds were prepared by modifications of published procedures (*1, 4*). Their toxicity was tested by injecting them intraperitoneally into male Swiss Webster mice (18 to 20 g), with LD_{50} (lethal dose, 50 percent effective) determinations being made after 24 hours. The administration vehicle was methoxytriglycol unless indicated otherwise.

The toxic 4-alkyl bicyclic phosphorus esters shown in Table 1 each produce convulsive seizures and death in mice within a few minutes. The bicyclic phosphites (P), phosphates [P(O)], and thionophosphates [P(S)] with the same 4-alkyl substituents are of similar toxicity, but the potency is greatly dependent on the nature of the 4-alkyl substituent with a maximum toxicity for compounds with the isopropyl group. Phosphates of the 4-isopropyl or 4-*n*-propyl compounds are slightly more toxic than the phosphites or thionophosphates. Bicyclic phosphites and thionophosphates undergo rapid and almost quantitative oxidation to the corresponding phosphates on treatment with *m*-chloroperoxybenzoic acid, as expected from earlier studies (*1, 4*). Comparable oxidation reactions occur with many other thionophosphates, including parathion, in living mammals (*5*). Accordingly, it is likely that the bicyclic phosphites and thionophosphates are oxidized in vivo prior to exerting their poisoning action.

The mode of action of toxic phosphorus esters has been extensively studied because of their importance as insecticides and chemical warfare agents, exemplified by parathion and diisopropyl fluorophosphate (DFP), respectively. Their toxicity is generally attributed to inhibition of acetylcholinesterase (AChE) in the nervous system (*5*). The toxic signs produced by 4-alkyl bicyclic phosphorus esters in mice and rats do not, however, resemble the characteristic manifestations of poisoning by anticholinesterase agents, that is, there is no indication of excessive parasympathetic stimulation, twitching of the muscles, paralysis, or clonic convulsions. Rats treated intraperitoneally with the isopropyl bicyclic phosphate at 0.3 mg/kg die in 20 to 30 minutes without inhibition of the AChE activity in the whole brain or blood (*6*). Even when the concentration of the isopropyl bicyclic phosphate is 3 mg/kg, which produces death within 5 minutes, the brain AChE activity is not at all inhibited. For comparison, rats 1 hour after treatment with a nonlethal dose

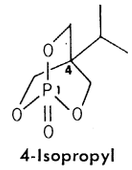
Table 1. Intraperitoneal toxicity to mice of bicyclic phosphorus esters in comparison with parathion and DFP; P, phosphite; P(O), phosphate; P(S), thionophosphate.

Type structure	LD_{50} (mg/kg)		
	X = P	X = P(O)	X = P(S)
Bicyclic phosphorus esters			
$NO_2C(CH_2O)_3X$		9.5	
$HOCH_2C(CH_2O)_3X$	> 500*	> 500†	> 500*
$CH_3C(CH_2S)_3X$			95*‡
$CH_3C(CH_2O)_3X$		32	34
$CH_3CH_2C(CH_2O)_3X$	1.1§	1.0	1.1
$CH_3(CH_2)_2C(CH_2O)_3X$	0.39	0.38	0.79
$(CH_3)_2CHC(CH_2O)_3X$	0.22	0.18‖	0.26
$(O)P(CH_2O)_3X$	174*‡	189*‡	
Parathion and DFP			
$4\text{-}NO_2\text{-}\emptyset OX(OCH_2CH_3)_2$			5.9
$FX[OCH(CH_3)_2]_2$		6.0	

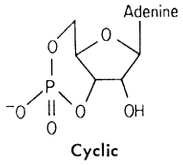
* Dimethyl sulfoxide was used as the vehicle. † Water was used as the vehicle. ‡ Samples were provided by Professor J. G. Verkade, Department of Chemistry, Iowa State University, Ames. § Other mouse LD_{50} values are 4 mg/kg dermally, with 10 μl of dimethyl sulfoxide as vehicle per mouse for treatment on the back; 7 mg/kg, orally, with 50 μl of methoxytriglycol for administration by stomach tube. ‖ This is the 4-isopropyl bicyclic phosphate (see structure).

of DFP, 3 mg/kg intraperitoneally, show signs of excessive cholinergic stimulation and a 54 percent inhibition of AChE activity in whole blood and an 87 percent inhibition in whole brain. While this enzyme assay might not detect reversible AChE inhibition, the symptoms of poisoning rule out the possibility that the isopropyl bicyclic phosphate acts in this manner. Barbiturates may be useful antidotes in combatting acute convulsions produced by the bicyclic phosphorus esters, as judged by preliminary studies involving intraperitoneal administration of phenobarbital to mice poisoned with the ethyl bicyclic phosphate.

Those who work with bicyclic phosphorus esters should use suitable precautions to avoid poisoning since some of these compounds, in mouse tests, are 33 times more toxic than DFP or parathion. They are not only highly toxic materials but they appear to poison by a mechanism different from that of any other known organophosphorus toxicants. While the mode of action of the bicyclic phosphorus esters is not as yet defined, their structural similarity to the cyclic phosphate adenosine 3′,5′-monophosphate (cyclic AMP) is possibly relevant. These compounds may be useful probes in pharmacological and biochemical studies.



4-Isopropyl bicyclic phosphate



Cyclic AMP

EUGENE M. BELLET*
JOHN E. CASIDA

Division of Entomology and Parasitology, University of California, Berkeley 94720

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* Present address: Office of Pesticide Programs, Environmental Protection Agency, Washington, D.C.

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Direct Evidence for a Colchicine-Induced Impairment in the Mobility of Membrane Components

Abstract. *Freeze-etch electron microscopy was used to show that colchicine interacts with membranes of the ciliate protozoan* Tetrahymena pyriformis. *Colchicine impairs the temperature-induced translational and vertical mobility of the membrane-intercalating particles of the freeze-fractured alveolar membranes lying just below the plasma membranes.*

Recent studies suggest that membranes may be envisaged as two-dimensional "fluid" or "plastic" entities that are assemblies of noncovalently bonded lipids and proteins (*1*). This would explain findings suggesting that diverse membrane components can move translationally and normally to the membrane plane (*2–4*). Such motion of membrane components has been recently implicated in the lectin-induced agglutination of various neoplastically transformed cells (*5*), as well as to the "cap" formations and redistributions of surface antigens in different cell types (*6*).

However, these membrane properties can be inhibited by colchicine, Colcemid, or vinblastine (*7*). Since these alkaloids disaggregate microtubules (*8*), microtubular proteins are now thought to influence the mobility of membrane components. On the other hand, a direct action of these agents on membranes, possibly causing a decreased motion of membrane components, cannot be excluded, particularly since colchicine also binds to isolated membrane fractions (*9*).

We have accordingly examined the possible direct action of colchicine on biomembranes by freeze-etch electron microscopy, since only this method can directly detect movements of membrane components at high resolution (about 20 Å). At fracturing, membranes are internally split, exposing two faces of their apolar membrane cores (*10*). Such membrane faces are generally studded with randomly distributed 85-Å particles, composed at least partially of proteins or glycoproteins (or both) (*11*) and bearing diverse antigenic specificities (*12*).

These membrane-intercalated particles can move translationally at least in some membranes as a function of *p*H (*3*) or temperature (*4*). We now analyze the influence of colchicine on particle movement induced by variations in temperature, using the membranes of the alveolar sacs lying just below the plasma membrane of the ciliate eukaryote *Tetrahymena pyriformis* (*4, 13*).

Early log-phase *Tetrahymena* cultures were grown at the optimal temperature of 28°C in a medium of 2 percent proteose peptone plus 0.4 percent liver extract. Cultures were divided into two portions; one was incubated with colchicine (5 mg/ml) and one used as control. The relatively high dose is not lethal for the cells; cell division is first blocked, but cell growth continues, and about 4 hours after incubation the cells even "adapt" to the drug (*14*). About 30 minutes after incubation with colchicine, both control and colchicinized cells were simultaneously cooled (cooling rate, about 6°C/min) from the optimum growth temperature of 28° to 5°C, kept at 5°C for 4 minutes, and then reheated to 28°C within 30 seconds, under the same conditions. For freeze-etch electron microscopy, cell samples were taken at 28°C just prior to chilling and immediately after reheating, and, during chilling, at 20°, 15°, 10°, and 5°C. The cells were fixed with 3 percent glutaraldehyde in 0.05*M* sodium cacodylate, *p*H 7.2 (about 10 minutes), washed, glycerinated in steps up to about 25 percent during a period of 2 to 3 hours, and frozen in Freon 22 on cardboard disks. Fracturing, etching (1 minute at −100°C), and replicating were performed in a Balzers machine (model BA 360 M; Balzers, Liechtenstein). Replicas were studied in a Siemens Elmiskop Ia.

At 28°C, in untreated cells the face of the outer fractured alveolar membranes oriented toward the plasma membrane [for closer description see (*4, 13*)] reveals in two experiments about 480 ± 75 particles and 640 ± 65 particles per square micrometer, respectively. The average distance between neighboring particles amounts to 44.9 ± 2.1 and 39.6 ± 1.9 nm, respectively (means of at least 100 measurements with standard error). These particles nearly always showed diameters larger than 100 Å. The average distance between the particles, as well as the frequency of the particles, diminish on cooling (Figs. 1 and 2). This phenomenon suggests a critical