PHOSPHOROUS CONTAINING ALKYLATING AGENTS:

CARCINOGENICITY AND STRUCTURE-ACTIVITY RELATIONSHIPS. OTHER BIOLOGICAL PROPERTIES. ACTIVATING METABOLISM. ENVIRONMENTAL SIGNIFICANCE.

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5.2.1.4 Phosphorus-Containing Alkylating Agents

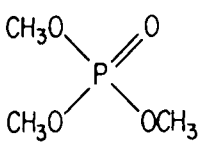
Synthetic organophosphorus compounds are of great economic importance. They have been extensively used for various practical purposes and are invaluable, if not indispensable, as pesticides, flame retardants and cancer chemotherapeutic agents. Thus, human exposure to these compounds may occur in a vast section of the population. Because of their wide use as pesticides, the toxicology of organophosphorus compounds has been extensively studied; there is, however, a relative scarcity of information on their carcinogenicity. Although organophosphorus compounds are generally regarded as inactive or as weak carcinogens, recent investigations revealed that some of them are potent agents. This section discusses the literature of organophosphorus compounds with special emphasis on their carcinogenicity. Because of the voluminous literature on the well-known chemotherapeutic agent, cyclophosphamide, a cyclic organophosphorus compound with a mustard functional group, this agent is reviewed separately in Section 5.2.1.4.2. Phosphoramides containing ethyleneimino groups have been previously discussed in Section 5.2.1.1.4.

5.2.1.4.1. Organophosphorus Compounds (Excluding Cyclophosphamide). Most of the compounds discussed in this subsection are derivatives of phosphoric acid and are often collectively termed organophosphates. The nomenclature of organophosphorus compounds is confusing; there is no simple, logical or generally accepted system. At least five or six different nomenclature systems have been used (see 1, 2). For the purpose of discussion in this subsection, the most common or generic names and abbreviations are used.

← Fig. 20

The structures of these compounds are depicted in Fig. 20 and their full chemical names following the Chemical Abstract or IUPAC system are listed in Table CVIII. Essentially the compounds discussed include (a) phosphoric acid triesters, $(RO)_3P{=}O$; (b) thiophosphoric acid triesters containing one thio group (phosphorothioate) or two thio groups (phosphorodithioate) [the term, phosphorothioate is a general term for phosphorothionate, $(RO)_3P{=}S$, and phosphorothiolate, $(RO)_2P{=}O(SR)$], (c) dialkyl phosphorofluoridate, $(RO)_2P{=}O(F)$, (d) phosphoric triamide, $(R_2N)_3P{=}O$, (e) dialkyl alkylphosphonate $(RO)_2P{=}O(R)$, and (f) tetraalkyl phosphonium chloride, $R_4P^{\oplus}Cl^{\ominus}$. Most of these compounds have been used as pesticides, whereas some have been employed as flame retardants (Tris-BP, THPC), nerve gas (DFP), chemosterilant (HMPA) and solvents or intermediates in organic synthesis of industrial chemicals (HMPA, TMP).

5.2.1.4.1.1 Physico-Chemical and Biological Properties. The physical and chemical properties of organophosphorus compounds have been extensively discussed in several excellent reviews (3-5) and monographs (1, 2, 6). Dichlorvos, in particular, has received great attention (3, 4) because of its extensive agricultural use. The physical constants of some of the widely used organophosphorus compounds are summarized in Table CVI. ← Table CVI The chemical reactivity of organophosphorus compounds is dependent on the chemical structure of the compound and reaction conditions such as the pH, the temperature, the solvent involved, and the presence of catalytic additives. Trimethyl phosphate is quite stable in neutral aqueous solution. Substitution of one of the methoxy groups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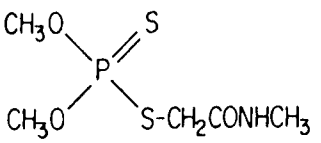
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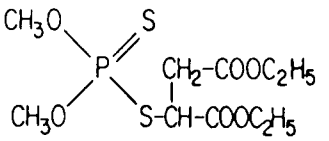
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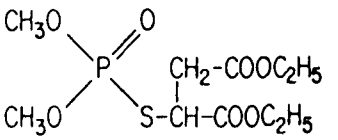
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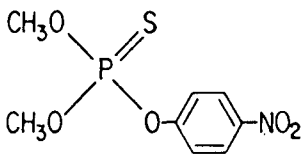
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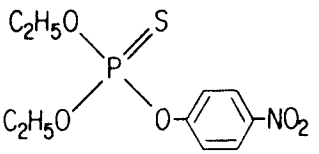
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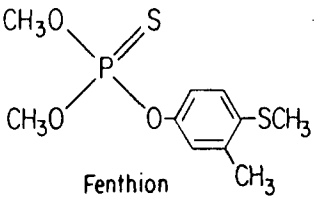
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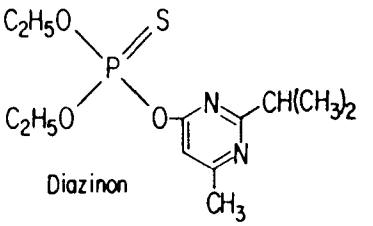
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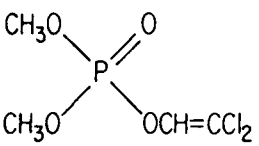
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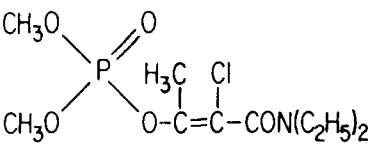
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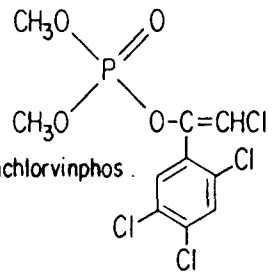
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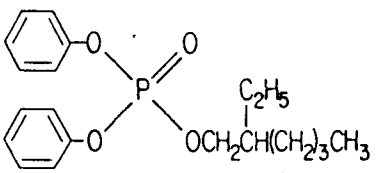
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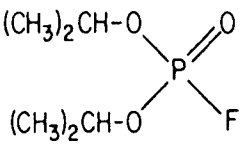
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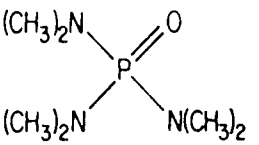
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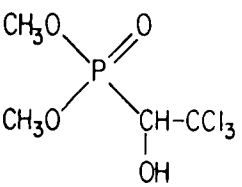
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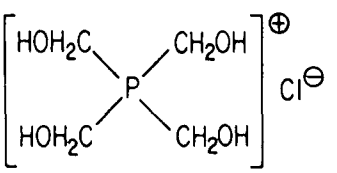
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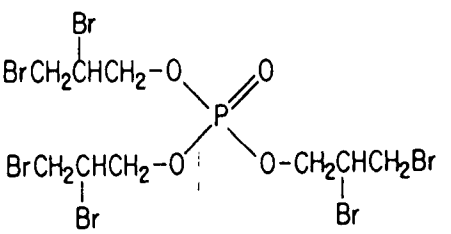

Tris-BP

Figure 20

LEGEND TO FIGURE 20

Fig. 20. Structural formulas of organophosphorus compounds that have been tested for carcinogenicity. (See Table CVIII for complete chemical names.)

Table CVI

Physical Constants of Some Widely Used Organophosphorus Compounds[a]

Compound	m. p.	b. p. at (mm Hg)	Density	Refractive index	Vapor pressure (mm Hg)	Solubility
Trimethylphosphate (TMP)	—	197.2°C	$d_0^{19.5}$ = 1.197	—	—	Freely soluble in water.
Dimethoate	52°C	107°C at 0.05 mm	—	—	8.5×10^{-6} at 20°C	Slightly soluble in water (3-4%); soluble in polar organic solvents.
Malathion	2.9°C	156-7°C at 0.7 mm	d_4^{25} = 1.23	n_D^{25} = 1.4985	4.0×10^{-5} at 30°C	Sparingly soluble in water (145 ppm); miscible with many organic solvents.
Methyl Parathion	37-8°C	109°C at 0.05 mm	d_4^{20} = 1.358	n_D^{25} = 1.5307	9.7×10^{-5} at 20°C	Sparingly soluble in water (50 ppm); soluble in most organic solvents.
Parathion	6°C	375°C	d_4^{25} = 1.26	n^{25} = 1.5370	0.003 at 24°C	Sparingly soluble in water (20 ppm); soluble in alcohols, esters, ketones.
Fenthion	—	87°C at 0.01 mm	d_4^{20} = 1.25	n_D^{20} = 1.5698	3.0×10^{-5} at 20°C	Sparingly soluble in water (55 ppm); soluble in alcohols and organic solvents.
Diazinon	—	83-4°C at 0.002 mm	d_4^{20} = 1.116-8	n^{20} = 1.4978	4.1×10^{-4} at 20°C	Sparingly soluble in water (40 ppm); miscible with alcohols and organic solvents.

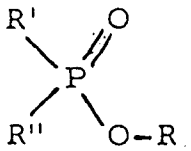
Dichlorvos	—	140°C at 20 mm	d_4^{25} = 1.415	n_D^{25} = 1.451	—	Slightly soluble in water (1%); miscible with organic solvents.
Phosphamidon	-45°C	162°C at 1.5 mm	d_4^{25} = 1.2132	n_D^{25} = 1.4718	2.5×10^{-5} at 20°C	Miscible with water and most organic solvents.
Tetrachlorvinphos	97-8°C	—	—	—	4.2×10^{-8} at 20°C	Sparingly soluble in water (11 ppm); soluble in most organic solvents.
Diisopropyl fluorophosphate (DFP)	-82°C	62°C at 9 mm	d = 1.035	n_D^{25} = 1.383	0.579 at 20°C	Slightly soluble in water (1.54%); soluble in vegetable oils.
Hexamethylphosphoramide (HMPA)	7.2°C	105-7°C at 11 mm	d_4^{20} = 1.03	n_D^{21} = 1.4572	—	Soluble in water, polar and nonpolar solvents.
Trichlorfon	83-4°C	100°C at 0.1 mm	d = 1.73	—	—	Soluble in water (15.4%), ether, chloroform and benzene.
Tris (2,3-dibromopropyl) phosphate (Tris-BP)	—	—	d^{25} = 2.27	—	1.9×10^{-4} at 25°C	Practically insoluble in water (1.6 ppm); soluble in acetone, dimethylformamide and organic solvents.

[a]Summarized from M. Eto, "Organophosphorus Pesticides. Organic and Biological Chemistry", CRC Press, Cleveland, 1974; "The Merck Index," 9th edn., Merck & Co., Rahway, NJ, 1976; J.A. Dean, "Lange's Handbook of Chemistry", 12th edn., McGraw-Hill, New York, 1979.

[b]See Fig. 20 for structural formulas and Table CVIII for complete chemical names.

by an electron-withdrawing ester group may increase susceptibility to hydrolysis, especially under alkaline conditions. The rate of hydrolysis of a number of organophosphorus pesticides (in a 1:4 mixture of ethanol and buffer, pH 6, at 70°C) follows the order: Dichlorvos ($t_{\frac{1}{2}}$ = 1.35 hr) > Trichlorfon (3.2 hr) > Malaoxon (7.0 hr) > Malathion (7.8 hr) > Methyl Parathion (8.4 hr) > Phosphamidon (10.5-14 hr) > Dimethoate (12 hr) > Fenthion (22.4 hr) > Paraoxon (28 hr) > Diazinon (37 hr) > Parathion (43 hr) (2). Amidophosphates (phosphoramides), on the other hand, have quite different hydrolytic properties; HMPA, for example, is quite stable in aqueous solution.

Organophosphorus compounds with the general structure $-\overset{\|}{\underset{|}{P}}-O-\overset{|}{\underset{|}{C}}-$ have two electrophilic sites where reaction with nucleophiles may take place. Nucleophilic attack at the phosphorus atom with subsequent cleavage of the P-O bond results in phosphorylation of the nucleophile, whereas a similar attack at the carbon atom brings about alkylation. The phosphorylation of serine residue at the "esteratic site" of cholinesterase, with subsequent irreversible inhibition, is universally regarded to be the mode of action of insecticidal organophosphorus compounds. This topic has been amply discussed by several authors (1, 2). The alkylating activity of organophosphorus compounds is believed to be the basis of their mutagenic, carcinogenic and other deleterious biological activities. The relationship between chemical structure and alkylating properties has been reviewed (1, 2). Methylester organophosphates are stronger alkylating agents than ethyl or higher n-alkyl ester derivatives. Phosphate esters are generally more reactive than the corresponding phosphorothionate esters. Substituents, R' and R'' play an important role in modifying

the alkylating activity of organophosphates of the type  . The alkylating activity of the R group positively correlates with the electron attracting capability of the substituents. Thus, alkylating activity decreases in the order: R' and/or R'' = p-NO_2-C_6H_4-O- > C_6H_5-O- > CH_3-S- > CH_3O- > CH_3-. A variety of organophosphorus compounds have been shown to exhibit alkylating activity as measured by the reaction with 4-(p-nitrobenzyl)pyridine (abbreviated as NBP). Preussmann et al. (7) semi-quantitatively rated Methyl Parathion, Malathion and Trichlorfon as strongly positive (+++) and Parathion as weakly positive (++) alkylating agents. A more detailed study by Bedford and Robinson (3) listed the relative alkylating activity of several organophosphorus compounds in the following decreasing order: Dichlorvos > Methyl Paraoxon > Tetrachlorvinphos > Methyl Parathion > Malathion > Trichlorfon >> Trimethylphosphate > Diethyl analog of Dichlorvos. Dichlorvos is about 70 times less active than dimethyl sulfate in the NBP reaction. Demethylated Dichlorvos (i. e., 2, 2-dichlorovinyl monomethyl phosphate), dimethyl phosphate and a number of dichloro metabolites of Dichlorvos are all inactive as alkylating agents. The alkylating activity of Dichlorvos, Methyl Parathion and Trichlorfon was confirmed by the study of Fischer and Lohs (8); also Dimethoate was shown to be an active alkylating agent by the NBP reaction.

The toxicity of organophosphorus compounds used as insecticides has been extensively investigated; Table CVII summarizes the acute toxicity data. ← Table CVII In general, the principal toxic actions of these compounds are due to the inhibition of cholinesterase, thus increasing the neurotransmitter, acetylcholine,

Table CVII

Acute Toxicity of Several Widely Used Organophosphorus Compounds

Compound[a]	Species and route	LD_{50} (mg/kg)[b]	Reference
Trimethylphosphate (TMP)	Mouse, oral	1,470; 3,610	22, 128
	Rat, oral	840; 2,000	128, 129
	Guinea pig, oral	1,142	129
	Rabbit, oral	1,275	129
Dimethoate	Rat, oral	215	130
	Rat, oral	147 (LP), 152 (HP)	131
	Rat, topical	400	130
	Wild bird, oral	7	132
Malathion	Mouse, oral	3,321	45
	Rat, oral	1,375 (M), 1,000 (F)	130
	Rat, oral	599 (LP), 140 (HP)	131
	Rat, i.p.	750, 900	133, 134
	Guinea pig, i.p.	500	135
	Dog, i.p.	1,400	136
Malaoxon	Rat, i.p.	25	134

Methyl Parathion	Mouse, oral	17	137
	Mouse, i.v.	13	137
	Rat, oral	14 (M), 24 (F)	138
	Rat, topical	67	138
	Rat, inhalation	LC_{50} = 200 mg/m^3 or 19.5 ppm for 1 hr., 120 mg/m^3 or 11.4 ppm for 4 hr.[c]	139
	Guinea pig, oral	417	137
Parathion	Mouse, oral	6, 25	52, 140
	Mouse, i.p.	9-10	141
	Rat, oral	5-30 (M), 1.75-6 (F)	52, 130, 140, 141
	Rat, oral	4.9 (LP), 37.1 (HP)	131
	Rat, topical	21 (M), 6.8 (F)	130
	Rat, inhalation	LC_{50} = 18 ppm or 214 mg/m^3 for 1 hr.[c]	142
Fenthion	Mouse, oral	125 (M), 150 (F), 200	143, 144
	Rat, oral	260 (M), 325 (F)	143
	Rat, topical	330	130
	Domestic bird, oral	6; 11	145

Table CVII continued

Diazinon	Rat, oral	108 (M), 76 (F)	130
	Rat, topical	900 (M), 455 (F)	130
	Chicken, s.c.	15	146
	Wild bird, oral	2	132
Dichlorvos	Mouse, oral	140	147
	Rat, oral	80 (M), 56 (F)	148
	Rat, topical	107 (M), 75 (F)	148
	Rat, s.c.	52	149
	Chicken, s.c.	20	146
	Dog, oral	483 (newborn), 1,090 (adult)	150
Phosphamidon	Rat, oral	23.5	130
	Rat, topical	143 (M), 107 (F)	130
	Wild bird, oral	2	132
Tetrachlorovinphos	Mouse, oral	1,600	151
	Rat, oral	1,100	152
	Chicken, oral	2,528	153
	Wild bird, oral	100	132
Diisopropyl fluorophosphate (DFP)	Mouse, topical	72	151
	Rat, oral or topical	8	154
	Monkey, i.v.	0.28	151

Hexamethyl-phosphoramide (HMPA)	Rat, oral	2,650 (M), 3,360 (F)	65
	Rabbit, topical	2,600	155
	Chicken, oral	835	156
Trichlorfon	Mouse, i.p.	500	157
	Rat, oral	400, 450	132, 157
	Rat, i.p.	225	157
	Guinea pig, i.p.	300	157
	Wild bird, oral	40	132
Tetrakis(hydroxy-methyl)phosphon-ium chloride (THPC)	Mouse, oral	400	158
Tris (2,3-dibromo-propyl) phosphate (Tris-BP or TRIS)	Rat, oral	5,400	159
	Rabbit, topical	>8,000	9

[a]See Table CVIII for complete chemical names and Fig. 20 for chemical structures.

[b]Except where specified, the values shown are lethal doses for 50% kill (LD_{50}) in mg/kg body weight. (M) = male, (F) = female, (LP) = low protein diet; (HP) = high protein diet.

[c]LC_{50} = lethal concentration for 50% kill within the exposure time period specified.

with resultant excessive stimulation of the central and peripheral nervous systems and somatic motor nerves. The clinical toxic symptoms include, in the order of appearance, cramping abdominal pain, lacrimation, salivation, muscle fasciculation, excessive bronchial secretion, extreme respiratory distress, coma, convulsion and death. Phosphoramides tend to have more selective action on peripheral tissues. The toxic effects of insecticidal organophosphorus compounds can be alleviated by treatment with the muscarinic blocker, atropine and related compounds. The toxicity of some organophosphorus compounds shows variations according to the species, sex or age of the animal and may be modified by the diet (see Table CVII). The toxicology of the flame retardant, Tris-BP, has been investigated by Kerst (9) and was found to have a relatively low acute toxicity.

The mutagenicity of organophosphorus compounds was investigated in a variety of test systems. Trimethylphosphate is definitely mutagenic in microorganisms, Drosophila, cultured mammalian cells and laboratory rodents, although its potency is very low, and high dosages or concentrations are usually required (rev., 10). In microbial systems, TMP was found to be weakly mutagenic in several mutant strains of Salmonella typhimurium (11-14), Escherichia coli (12, 15), Klebsiella pneumoniae (16), Serratia marcescens (15), Pseudomonas aeruginosa (11), Neurospora crassa (17), and Saccharomyces cerevisiae (18). In some of these studies, a mammalian hepatic activation system is included; however, it has not been thoroughly investigated whether the inclusion actually enhances or diminishes the mutagenic effect of TMP.

It is important to note that TMP is capable of inducing dominant lethal mutations in several strains of mice (19-22). Although the effect is observed only at high doses (around 1 g/kg), it can be clearly demonstrated and appears to be dose-dependent The literature on mutagenicity of organophosphorus insecticides has been reviewed by Wild (23) The induction of mutation in microorganisms in *in vitro* systems has been unequivocally shown with Dichlorvos (11, 15, 16, 24-29), Dimethoate (23, 26-28), and Methyl Parathion (27, 28) at millimolar or higher concentrations Malathion, Parathion and Diazinon, however, appear to be inactive in several tests (15, 23, 24, 27, 28). In general, the *in vitro* mutagenic activity of these and several other organophosphorus compounds appears to parallel their alkylating activity as measured by the NBP reaction (18, 25). In contrast to its *in vitro* activity, Dichlorvos has been consistently found to be inactive in various *in vivo* tests including host-mediated assays (25, 30), chromosome aberration, micronucleus, and dominant lethal tests in experimental animals (19, 31-33). The apparent lack of activity in *in vivo* systems is believed to be due to the extremely rapid breakdown of the compound to nonalkylating metabolites and the impossibility to administer high doses because of toxicity The mutagenic effect of other organophosphorus insecticides in *in vivo* systems has not been experimentally investigated. Cytogenetic analysis of humans exposed to organophosphorus compounds such as Malathion, Methyl Parathion and Trichlorfon, did not indicate any significant or clear-cut increase in chromosomal aberration (34, 35). The mutagenicity of HMPA has also been studied, in Ames' Salmonella test, the compound gave erratic responses in a manner similar to that of dimethylnitrosamine (36). In

a cell transformation assay, however, HMPA consistently increased the transformation frequency of cultured mammalian cells after metabolic activation (36, 37) The recent discovery of the highly potent mutagenicity of Tris-BP has stirred great concern about its safety as the main flame retardant in children's sleepwear Tris-BP has been found to be mutagenic in the Salmonella test by several laboratories (38-41). The compound is active as such, however, the inclusion of metabolic activating system (S-9 mix) considerably enhances the mutagenic effect Actually, Tris-BP is a more potent mutagen than such well known human carcinogens as benzidine and β-naphthylamine (40)

5 2 1 4 1 2 Carcinogenicity and Structure- -Activity Relationships. Despite extensive studies of the acute toxicity of organophosphorus compounds, their potential carcinogenic activity has largely been neglected until recently Close to 20 organophosphorus compounds have been tested or are being tested, at the time of this writing, in the Carcinogenesis Testing Program of the National Toxicology Program The results of the completed studies and those of various other investigators are summarized in Table CVIII. ← Table CVIII

The unsubstituted trialkyl phosphate, TMP, has been found to be a weak carcinogen in rodents Oral administration of the compound (3 times/week for 2 years) to Fischer 344 rats (50 or 100 mg/kg/dose) or $B6C3F_1$ mice (250 or 500 mg/kg/dose) led to the induction of benign fibromas in the subcutaneous tissue in male rats and malignant adenocarcinomas of the uterus/endometrium in female mice (18% in the low-dose and 38% in the high-dose group) The effects were statistically significant However, no evidence of carcinogenicity of the compound in female rats or male mice was found (42)

Table CVIII

Carcinogenicity of Organophosphorus Compounds

Compound	Species and strain	Principal organs affected (Route)	Reference
Trimethylphosphate (TMP)	Mouse, $B6C3F_1$	Uterus (oral)	42
	Rat, F344	Subcutaneous tissue (oral)	75
O, O-Dimethyl S-(N-methyl-carbamoylmethyl) phosphorothioate (Dimethoate)	Mouse, AB	Hematopoietic system, mammary gland (topical)	43
	Mouse, $B6C3F_1$	None (oral)	44
	Rat, Wistar	Spleen, forestomach, lung (oral or i.m.)	43
	Rat, Osborne-Mendel	None (oral)	44
O, O-Dimethyl S-(1,2-dicarboxyethyl) phosphorothioate (Malathion)	Mouse, $B6C3F_1$	No significant effect (oral)	48
	Rat, unspecified	None (oral)	45
	Rat, Sprague-Dawley	Mammary gland (oral) (inconclusive)	46
	Rat, Osborne-Mendel	No significant effect (oral)	48
	Rat, F344	None (oral)	49
O, O-Dimethyl S-(1,2-dicarboxyethyl) phosphate (Malaoxon)	Mouse, $B6C3F_1$	None (oral)	50
	Rat, F344	None (oral)	50

O, O-Dimethyl O-(4-nitrophenyl) phosphorothioate (Methyl Parathion)	Mouse, $B6C3F_1$	None (oral)	51
	Rat, F344	None (oral)	51
O, O-Diethyl O-(4-nitrophenyl) phosphorothioate (Parathion)	Mouse, $B6C3F_1$	None (oral)	54
	Rat, albino	None (oral)	52, 53
	Rat, unspecified	None (oral)	Lehman, 1965 cited in ref. 54
	Rat, Osborne-Mendel	Adrenal gland (oral) (marginal)	54
O, O-Dimethyl O-[4-(methylthio)-m-tolyl] phosphorothioate (Fenthion)	Mouse, $B6C3F_1$	Skin, subcutaneous tissue (oral)	55
	Rat, unspecified	None (oral)	Douell et al., cited in ref. 55
	Rat, F344	None (oral)	55
O, O-Diethyl O-(2-isopropyl-6-methyl--4-pyrimidyl) phosphorothioate (Diazinon)	Mouse, $B6C3F_1$	None (oral)	160
	Rat, Carworth Farm albino	None (oral)	56
	Rat, F344	None (oral)	160
O, O-Dimethyl 2, 2-dichlorovinyl phosphate (Dichlorvos, DDVP)	Mouse, $B6C3F_1$	None (oral)	60
	Rat, unspecified	None (oral)	58
	Rat, Carworth Farm E	None (inhalation)	59
	Rat, Osborne-Mendel	None (oral)	60

O, O-Dimethyl 2-chloro-2--diethyl-carbamoyl-1--methyl-vinyl phosphate (Phosphamidon)	Mouse, $B6C3F_1$	None (oral)	57
	Rat, Carworth Farm N	None (oral)	61
	Rat, Osborne-Mendel	Spleen (oral) (marginal)	57
O, O-Dimethyl 2-chloro--1-[(2, 4, 5-trichloro)-vinyl] phosphate (Tetrachlorvinphos)	Mouse, $B6C3F_1$	Liver (oral)	62
	Rat, Osborne-Mendel	Thyroid, adrenal glands (oral)	62
2-Ethylhexyl diphenyl phosphate	Rat, Carworth Farm albino	No significant effect (oral)	63
Diisopropyl fluorophosphate (DFP)	Rat, Wistar	Pituitary gland (i. m.)	64
Hexamethylphosphoramide (HMPA, Hexamethylphoric triamide)	Rat, Sherman	None (oral)	65
	Rat, Charles River CD	Nasal cavity (inhalation)	66, 67
O, O-Dimethyl 1-hydroxy--2, 2, 2-trichloroethyl phosphate (Trichlorfon, Dipterex)	Mouse, AB	Forestomach, liver, hematopoietic system (topical)	43, 70
	Rat, BD	Local sarcoma (s. c.)	69
	Rat, Wistar	Forestomach (oral or s. c.)	70
		Spleen, forestomach, lung, ovary (oral or i. m.)	43

Tetrakis(hydroxymethyl)-phosphonium chloride (THPC)	Mouse, Swiss ICR/Ha	No significant effect (topical)	72
Tris-(2,3-dibromo-propyl)-phosphate (Tris-BP, TRIS)	Mouse, Swiss ICR/Ha	Lung, forestomach, oral cavity, skin (topical)	72
	Mouse, $B6C3F_1$	Kidney, lung, liver, stomach (oral)	75
	Rat, F344	Kidney (oral)	75

Close to a dozen organophosphorus insecticides have been tested for carcinogenicity. In general, they have been found to be inactive or weakly active carcinogens. Dimethoate is one of the few organophosphorus insecticides that exhibit carcinogenic activity. Gibel et al. (43) reported that 15 of 101 Wistar rats of either sex given Dimethoate (5, 15 or 30 mg/kg, p.o. or 15 mg/kg, i.m., twice weekly for 10 weeks) developed malignant tumors (compared to none in controls). The malignancies were mainly reticulum cell sarcomas of the spleen and malignant reticulosis. In addition, substantial increases in the incidences of benign tumors, particularly in the forestomach and the lung, were observed. By topical application to AB mice, Dimethoate induced malignancies in 5 of 19 mice —— 4 leukemia and 1 mammary adenocarcinoma (43). In contrast to the above finding, however, a more recent study reported by the National Cancer Institute (44) did not confirm the carcinogenicity of the compound. In this study, Osborne-Mendel rats and $B6C3F_1$ mice were fed Dimethoate at doses ranging from 155 to 500 ppm for up to 115 weeks; no increases in tumor incidence attributable to the pesticide were observed (44).

The chronic toxicity of Malathion was investigated by Hazleton and Holland (45). Rats of an unspecified strain were fed diets containing 100, 1000 or 5000 ppm of either 65, 90 or 99% pure preparation of the compound. No tumors associated with the administration of the compound were observed. Only indirect and inconclusive evidence of possible carcinogenicity of Malathion has been provided (46); Sprague-Dawley rats were given a dose of 15 mg dimethylbenzanthracene, a mammary carcinogen, and kept on diets containing either 250 ppm or no Malathion. The Malathion-treated rats showed a higher

incidence of mammary tumors and shorter induction time (1.31 0.18 tumors/rat; 20 days to the appearance of the first tumor) than controls (1.07 0.15 tumors/rat; 25 days to first tumor). It is not known whether the enhancement of the carcinogenic effect reflected the modifying role of the pesticide or its carcinogenic effect acting synergistically or additively with dimethylbenzanthracene. In several industry-sponsored studies reported to the National Institute of Occupational Safety and Health (47) Malathion was found to be noncarcinogenic in albino rats after feeding for 2 years. The lack of carcinogenicity of Malathion has been confirmed in several recent National Cancer Institute studies. No clear evidence of association of tumor induction with Malathion could be demonstrated in Osborne-Mendel rats (fed diets containing 4700 or 8150 ppm of the compound for 80 weeks), F344 rats (2,000 or 4,000 ppm for 105 weeks), or $B6C3F_1$ mice (8,000 or 16,000 ppm for 80 weeks) (48, 49). Malaoxon, the activated metabolite of Malathion, has also been tested and found to be noncarcinogenic after feeding for 103 weeks to F344 rats and $B6C3F_1$ mice. The doses administered (500 or 1,000 ppm for both rodents) were considerably lower than those of Malathion because of the substantially higher acute toxicity of the metabolite (50).

Four dialkyl aryl phosphorothiates, Methyl Parathion, Parathion, Fenthion and Diazinon, have been tested for carcinogenicity. Among these, Methyl Parathion has been found to be noncarcinogenic in F344 rats and $B6C3F_1$ mice. The doses administered were 20 or 40 ppm in the diet for 105 weeks for the rats and 62.5 or 125 ppm for 102 weeks for the mice (51). The carcinogenicity of Parathion has been evaluated by several groups of investigators.

In a 2-year chronic toxicity study of the compound, Hazleton and Holland (52) did not observe, in male albino rats, any increase in tumor incidence attributable to the administration of up to 100 ppm of the compound in the diet. A similar observation was reported by Barnes and Denz (53) who fed albino rats for one year, diets containing 10-100 ppm of the compound. A 2-year study by the Food and Drug Administration also indicated no carcinogenic effect in an unspecified strain of rats (Lehman, 1965, cited in Ref. 54). In the recent NCI study, $B6C3F_1$ mice also did not develop tumors after receiving 80 or 160 ppm of the pesticide in the diet for 71 weeks. In the Osborne-Mendel rat, however, there was a higher incidence of cortical tumors (mostly adenomas) of the adrenal gland after administration of diets containing 23-63 ppm of Parathion for 80 weeks. The increase in tumor incidence was statistically significant and dose-dependent when compared to pooled or historical controls, but insignificant when compared to matched controls, probably because of the small number of rats used (males: pooled control 3/80, matched control 0/9, low-dose 7/49, high-dose 11/46; females: pooled control 4/78, matched control 1/10, low-dose 6/47, high-dose 13/42). The results are considered to suggest a possible (but not conclusive) association between tumor induction in the adrenal gland and Parathion administration, in this particular strain of rats (54). The carcinogenicity of Fenthion has also been tested at the National Cancer Institute by feeding F344 rats and $B6C3F_1$ mice for 103 weeks, diets containing 10 or 20 ppm of the pesticide. No increase in tumor incidence was observed in the rat. Female mice also did not develop tumors related to the pesticide treatment. In male mice, however, significant increases in the incidence

of sarcomas, fibrosarcomas and especially rhabdomyosarcomas of the integumentary system were observed, suggesting carcinogenicity of this compound (55). In a previously unreported study, Doull and associates did not detect any increase in tumor incidence after feeding rats for 1 year, diets containing up to 100 ppm of Fenthion (Doull et al., cited in ref. 55). The substitution of the p-nitrophenyl group of Parathion by a substituted pyrimidine ring (yielding Diazinon) does not seem to confer any carcinogenicity. Bruce et al. (56) observed no tumors in Carworth Farm albino rats after feeding for 72 weeks diets containing 10-1,000 ppm Diazinon. The lack of carcinogenicity of Diazinon has been confirmed by a recent National Cancer Institute study in which F344 rats and $B6C3F_1$ mice were fed diets containing 400 or 800 ppm (for rats) and 100 or 200 ppm (for mice) of the compound, for 103 weeks (57). Thus, with a few exceptional cases, dialkyl aryl phosphorothioates are mostly inactive as carcinogens.

Among the organophosphorus compounds, dialkyl vinyl phosphates are considered active alkylating agents and are, therefore, suspected to be potential carcinogens. The carcinogenicity of Dichlorvos has been extensively investigated; it appears, however, that there is no evidence that the compound is carcinogenic in experimental animals. The lack of carcinogenicity of Dichlorvos was reported in a Food and Agriculture Organization/World Health Organization Joint Meeting in 1966. Rats of an unspecified strain were fed nominal concentrations of 0.1, 1, 10, 100 and 500 ppm Dichlorvos in food for 2 years; there was no indication that the compound led to any increase in the tumor incidence (58). A 2-year inhalation carcinogenesis study has been conducted for

Dichlorvos using Carworth Farm E strain rats The animals were exposed to atmospheres containing 0 05, 0 5 and 5 0 mg/m^3 Dichlorvos continuously for up to 2 years There was no compound-related increase in tumor incidence in rats of either sex (59) In a National Cancer Institute study, Osborne-Mendel rats and $B6C3F_1$ mice were given technical grade Dichlorvos in the diet at two dose levels 150 ppm for 80 weeks or 1,000 ppm for 3 weeks followed by 300 ppm for 77 weeks There was no statistically significant increase in the incidence of tumors attributable to exposure to Dichlorvos in either animal species (60). Phosphamidon, another dialkyl vinyl phosphate, was also shown to have no carcinogenic effect in Carworth Farm N strain rats The animals received, in the diet, 0 1-5 mg/kg Phosphamidon daily for two years (61). In the National Cancer Institute study, Osborne-Mendel rats and $B6C3F_1$ mice received 80 or 160 ppm Phosphamidon in the feed for 62-80 weeks There was no increase in tumor incidence in the mouse of either sex In the male rats, the combined incidence of hemangiomas and hemangiosarcomas in the spleen showed a dose-related increase In the female rats, increases in C-cell adenoma and carcinomas of thyroid were observed These increases were statistically significant compared to pooled controls but insignificant compared to matched controls The data are considered marginal and insufficient to establish an association between tumor induction and the compound (57) In contrast to the above two compounds, there are strong indications for the carcinogenicity of Tetrachlorvinphos In a National Cancer Institute study, Osborne--Mendel rats were fed Tetrachlorvinphos for 80 weeks at the dose of 4,250 or 8,500 ppm, whereas $B6C3F_1$ mice were given 8,000 or 16,000 ppm of the

compound for 80 weeks There were significant dose-related increases in the proliferative lesions of the C-cells of the thyroid and cortical adenoma of the adrenals in female rats. In male mice, Tetrachlorvinphos was clearly carcinogenic causing significant increases in the induction of hepatocellular carcinomas (0/9 in controls, 36/50 in low-dose group, 40/50 in high-dose group). In female mice, the increase in hepatocellular carcinoma was not statistically significant, although substantial increases in the incidence of neoplastic nodules of the liver were observed (62)

The carcinogenicity of 2-ethylhexyl diphenylphosphate, a monoalkyl diaryl phosphate, has been tested by Treon et al (63). Carworth albino rats fed 1% or less of the compound in the diet for 2 years did not develop tumors attributable to the administration Limited studies were also carried out using several dogs, there was no sign of carcinogenicity (63)

In a study originally designed to investigate the behavioral effect of diisopropyl fluorophosphate (DFP), Glow (64) noted the induction of pituitary tumors in female Wistar rats Out of 100 rats, 16 confirmed cases of pituitary tumors were recorded This figure did not include those animals that died or were killed because of other causes The animals were administered an initial dose of 1 mg/kg followed by booster doses of 0 5 mg/kg every 72 hours The average induction time was 12-18 months It should be noted that the spontaneous occurrence of pituitary tumors is extremely rare in Wistar rats. The author (64) suggested that DFP could have a specific oncogenic effect on the pituitary gland of female Wistar rat

The carcinogenicity of hexamethylphosphoramide (HMPA) has been tested by two different routes of administration, with entirely different results. Oral administration of the compound to Sherman strain rats at the doses of 6.25, 3.12, 1.57 and 0.78 mg/kg/day for 2 years did not elicit any significant carcinogenic effect (65). By the inhalation route, however, HMPA has been found to be an extremely potent carcinogen in the Sprague-Dawley rat (66, 67). The induction of tumors in the nasal cavity (a site with extremely low spontaneous tumor incidence) was observed after exposure (5 hr/day, 5 days/week for 1 year) to HMPA in concentrations as low as 50 ppb (67). The carcinogenic effect was clearly dose-dependent. The time to the appearance of the first tumor was 6-7 months for the 4,000- and 400-ppb groups, 9 months for the 100-ppb group, and 13 months for the 50-ppb group; nasal tumors were not observed in the 10-ppb group (67). The nasal tumors were predominantly squamous cell carcinomas and occasionally, squamoadenocarcinomas or papillomas. Some tumors metastasized to the cervical lymph nodes and lungs, or invaded directly into the brain (66, 67).

The carcinogenicity of phosphoramides is probably not uniquely associated with HMPA; other members of the class may also possess carcinogenic activity. Ashby et al. (37) have evaluated the carcinogenic potential of various derivatives of HMPA by in vitro cell transformation assay of Styles (68). ← Fig. 21 The results are summarized in Fig. 21. Hexamethylphosphoramide (i) has been found to substantially increase the transformation frequency of cultured BHK 21/C13 cells after metabolic activation by the standard rat liver homogenate activation system (S-9 mix). Among the derivatives tested, hexamethylphosphorus triamide (ii) and hexamethylphosphorothioic triamide (iii) were

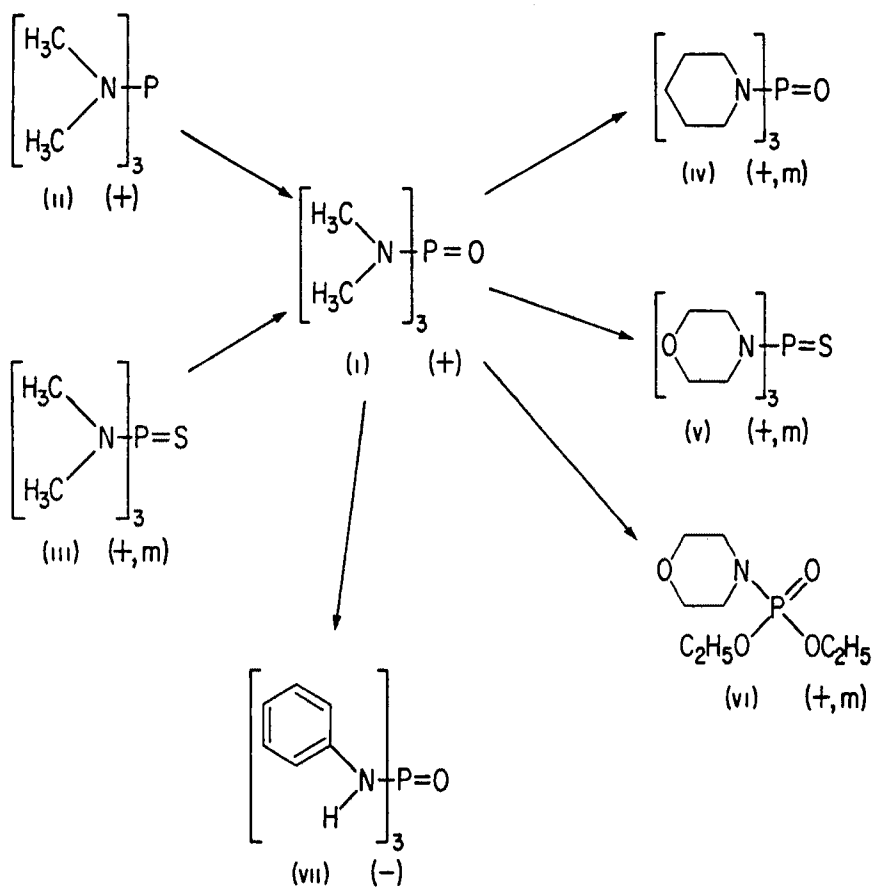


Figure 21

LEGEND TO FIGURE 21

Fig. 21. Structural formulas of the results of cell transformation assays obtained with hexamethylphosphoramide (HMPA) derivatives. The chemical names of the compounds are: *i* = hexamethylphosphoramide; *ii* = hexamethylphosphorous triamide; *iii* = hexamethylphosphorothioic triamide; *iv* = tripiperidinophosphine oxide; *v* = phosphorothioic trimorpholide; *vi* = diethoxymorpholinophosphine oxide; *vii* = phosphoric trianilide. The results of the cell transformation assays are designated as follows: "+" = positive after addition of standard S-9 mix; "+, m" = positive after addition of modified S-9 mix; "-" negative.

also active, suggesting that the $-\overset{|}{\underset{|}{P}}=O$, $-\overset{|}{\underset{|}{P}}$ and $-\overset{|}{\underset{|}{P}}=S$ groups may be considered metabolically equivalent because of the ready oxidation of the latter two groups to the former. Three alicyclic analogs of HMPA, tripiperidinophosphine oxide (iv), phosphorothioic trimorpholide (v), and diethoxymorpholinophosphine oxide (vi), have been found inactive after incubation with the standard S-9 mix. However, using a modified S-9 mix (containing higher proportion of S-9 tissue fraction in the mix) cell-transforming activity has been clearly demonstrated. Thus, these three compounds are regarded as potentially carcinogenic but possessing a much lower potency than (i). In contrast to the above compounds, phosphoric trianilide (vii), which lacks hydrogen on the carbon atom alpha to the nitrogen, is completely inactive. These results are supportive of a suggestion made earlier by Ashby _et al._ (36) that there is a structural analogy between phosphoramides ($>N-\overset{|}{\underset{|}{P}}=O$) and nitrosamines ($>N-N=O$) and that the relative structure-activity relationships of the latter class of carcinogens may be of help in predicting the potential carcinogenicity of compounds in the former class. Further studies are needed to establish whether the conclusions reached from cell transformation assays are applicable to _in vivo_ carcinogenesis assays.

Trichlorfon is the only alkyl phosphonate pesticide that has been tested for carcinogenicity. It has been found carcinogenic in rodents by several investigators. By s.c. injection to BD rats, a dose of 50 mg/kg induced local sarcomas in 2/24 animals (69). Topical application to AB mice led to the induction of one liver carcinoma and one forestomach papilloma out of 16 animals in one study (70), and myeloid leukemia in 5/14 mice in another study

(43). In the Wistar rat, oral or s. c. administration of Trichlorfon elicited the development of papillomas in the forestomach of 3/52 or 7/61 rats, respectively. Extensive liver necrosis and cirrhosis were also observed (70). In a more recent study, Wistar rats were given Trichlorfon orally or intramuscularly. Eleven of the 55 rats developed a variety of malignant tumors (compared to zero in controls); close to 50% (26/55) of the animals developed benign tumors. The malignancies were mainly reticulum cell sarcomas of the spleen and malignant reticulosis, whereas benign tumors were mostly found in the forestomach, the lung and the ovary (43).

The carcinogenic potential of chemical flame retardants has been a subject of great concern because of their extensive use in children's sleepwear and the discovery of potent mutagenicity of some of these compounds. Two organophosphorus flame retardants, THPC and Tris-BP, have been tested. The concern over THPC stems from the fact that the compound may break down into phosphine (PH_3), formaldehyde and hydrochloric acid. The latter two products may in turn combine to form the potent carcinogen, bis(chlormethyl) ether (see Section 5.2.1.1.2.7). In a preliminary mouse skin experiment, application of THPC (2 mg in 0.1 ml dimethylsulfoxide, 3 times/week for 400 days) to 20 female ICR/Ha Swiss mice led to the induction of one squamous carcinoma of the skin (71). In a subsequent experiment with a larger group (60) of animals at the same dose, but with acetone:water (9:1) as the solvent, no significant increase in the tumor incidence could be observed (72). The difference in the results from these two experiments was attributed to the solvent

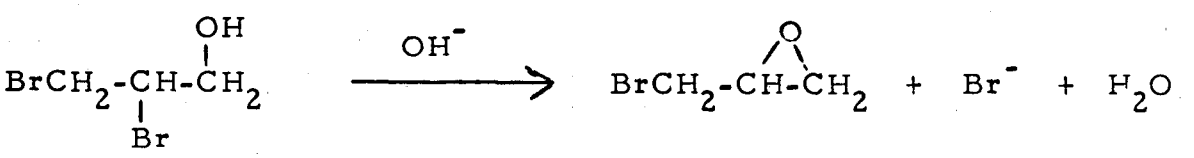
(72) as dimethylsulfoxide has been reported to have an unusual effect in mouse skin carcinogenesis studies (73).

Tris-(2, 3-dibromopropyl)phosphate was first reported to be carcinogenic in rodents by the Consumer Product Safety Commission (74) based on the preliminary results of a National Cancer Institute study. The study has now been completed and published (75). Fischer 344 rats and $B6C3F_1$ mice were given technical grade Tris-BP in the feed for 103 weeks at doses of 50 or 100 ppm for rats and 500 or 1,000 ppm for mice. Among mice, a variety of tumors arose as a result of Tris-BP administration; these included renal tubular cell adenoma and carcinoma, squamous cell adenoma and carcinoma of the forestomach, hepatocellular adenoma and carcinoma, and bronchiolar/alveolar adenoma and carcinoma. The incidences of renal adenoma, gastric adenoma, and pulmonary carcinoma were all significantly higher in dosed male mice than controls mice, whereas in dosed female mice significant increases in the incidence of hepatocellular adenoma and carcinoma, gastric adenoma and carcinoma, and pulmonary tumors were observed. In the rat, renal tubular adenomas were also observed at incidences which were significantly higher in dosed rats of both sexes than in the controls. For male rats, there was also a positive association between renal tubular cell adenocarcinoma and Tris-BP treatment. The possibility that the above carcinogenic effects might be due to 1,2-dibromo-3-chloropropane, a common carcinogenic contaminant of Tris-BP, was considered. After comparing the types and incidences of tumors induced by the contaminant (see Section 5.2.2.1) and Tris-BP, it was concluded that the effects observed were principally due to Tris-BP itself (75). In another

recent study (72), Tris-BP has been shown to be an active carcinogen inducing a variety of local as well as distant tumors in female ICR/Ha Swiss mice after skin application (3 times/week for 420-496 days) at two dose levels. In the high dose group (30 mg/application), the tumor incidences (out of 30 animals) in various organs were: dorsal skin 5 (3 papillomas, 1 carcinoma, 1 sarcoma), lung 28 (papillary tumors), liver 1 (carcinoma), forestomach 20 (13 papillomas, 7 carcinomas), oral cavity 4 (2 carcinomas each in the tongue and gingiva), and kidney 1 (tubular adenoma). Essentially the same targets were affected in the low-dose group (10 mg/application) but with lower incidences. The incidences in all the above targets were significantly higher than those of control mice. The time of the appearance of the first stomach carcinoma was as short as 21 weeks. The induction of tumors in the oral cavity and gingiva was thought to be related to the grooming habit of the mice (72).

The relationship between the chemical structure and carcinogenic activity of organophosphorus compounds has not been systematically investigated. From the available carcinogenicity data, it seems evident that the carcinogenicity of an organophosphorus compound is not solely dependent on its chemical structure. Biological factors such as activation and biodegradation play important limiting roles. To be carcinogenic, an organophosphorus compound must possess alkylating activity and also be able to reach target tissues in an active form. Thus, despite being one of the most active alkylating agents (among organophosphorus compounds), Dichlorvos is not carcinogenic because of its extremely short lifetime in biological systems (see "Metabolism"). On the other hand, TMP, a very weak alkylating agent, exerts a carcinogenic

effect at high doses because of its slow metabolism. The structural features that render several of the organophosphorus compounds carcinogenic possibly obey the following rationale. Tetrachlorvinphos may be carcinogenic because the elctron-withdrawing properties of the vinyl and aryl moieties make the cleavage of the H_3C-O bond easier thus enhancing the alkylating activity of the methyl group. Although the alkylating activity of tetrachlorvinphos is not as strong as dichlorvos, tetrachlorvinphos may, because of its relatively lower susceptibility to alkaline hydrolysis (3), have a better chance of reaching the target tissue. For DFP, the presence of the strongly electronegative fluorine makes the departure of the isopropyl group easier. Stabilization by the inductive effect of fluroine renders monoisopropyl fluorophosphate a good leaving group. Trichlorfon may be considered a precursor of dichlorvos (see "Metabolism"); it is possible that Trichlorfon has a greater probability of gaining access to the target tissue before being degraded. In the case of Tris-BP, the presence of the electronegative bromine atoms should make the compound a good alkyl donor possibly through displacement type of reaction. Alternatively, the cleavage of either C-O or O-P bond may generate 2,3-dibromopropanol which could, under mildly alkaline condition, generate highly reactive epoxide intermediate.



5.2.1.4.1.3 Metabolism and Possible Mechanism of Action. Organophosphorus compounds undergo, in virtually every biological system, extensive metabolic transformations that can bring about either activation or detoxication. Extensive reviews of this subject have been undertaken by various authors in recent years (2, 4-6, 76-79). Essentially, the activating pathways, as depicted in Fig. 22, Fig. 22 include (i) oxidative desulfuration of the thionophosphorus moiety (P=S) to the corresponding oxon (P=O) analogs, (ii) sulfoxidation of a thioether moiety present in the ester group to the respective sulfoxides or sulfones, (iii) N-dealkylation of the amide moiety of phosphoramide or organophosphate-containing amide structure, and (iv) thiono-thiolo rearrangement (isomerization) which transforms phosphorothionate to the more reactive phosphorothiolate. The former three reactions are catalyzed by the microsomal mixed-function oxidase present in mammalian cells, whereas the thiono-thiolo rearrangement may be triggered enzymatically or may occur nonenzymatically. It is not known whether the above-mentioned activation pathways, derived mainly from studies dealing with activation of insecticidal activity and acute toxicity, also apply to the generation of potential carcinogenic (presumably alkylating) intermediates. However, as discussed in the section on physico-chemical properties, modification of the chemical structure, known to increase the positive charge on the phosphorus atom (e.g., thiono → oxon, thiono → thiolo transformations), can also increase the alkylating activity of the organophosphorus compound. The detoxication pathways of the organophosphorus compounds are of great variety in mammals; they include (a) hydrolysis of the phosphoric ester linkage, (b) hydrolysis of carboxyester (e.g., Malathion) or carboxamide

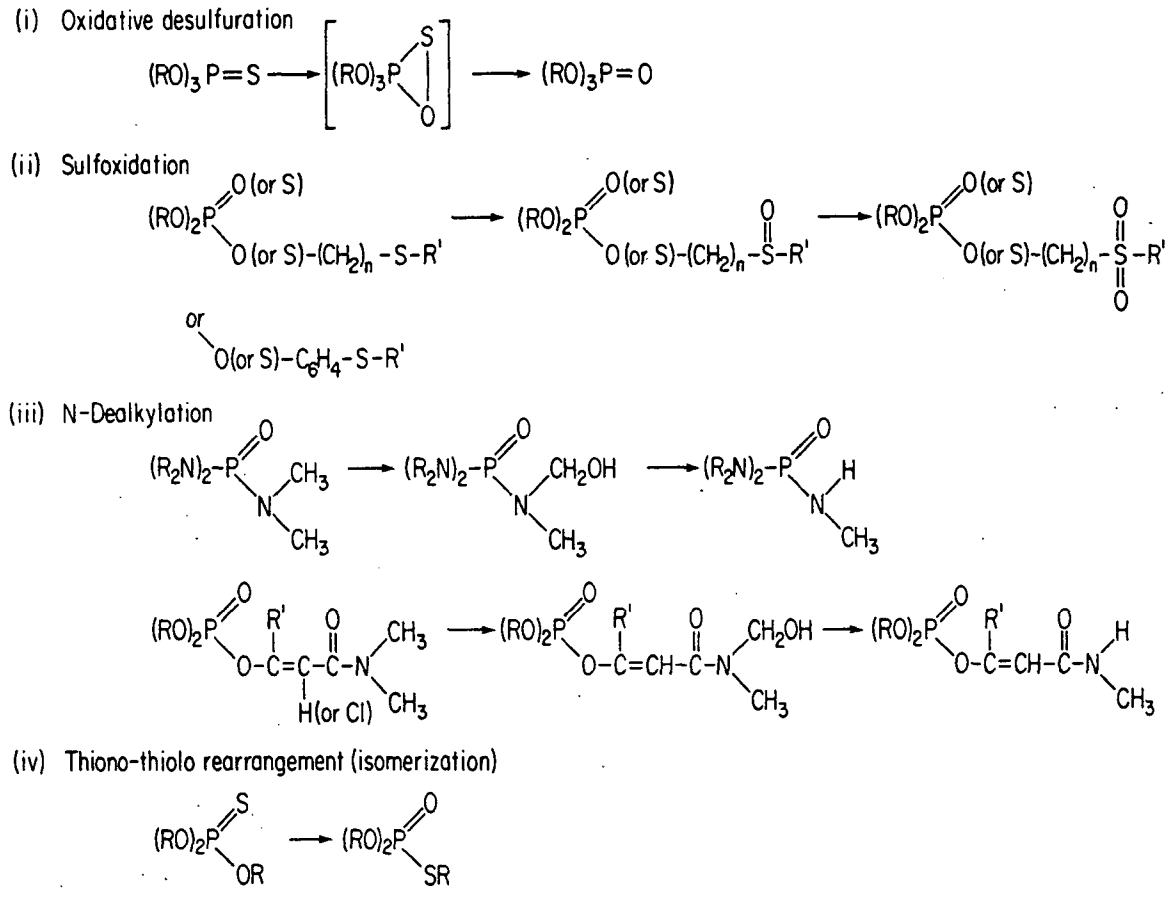

(i) Oxidative desulfuration
(ii) Sulfoxidation
(iii) N-Dealkylation
(iv) Thiono-thiolo rearrangement (isomerization)

Figure 22

LEGEND TO FIGURE 22

Fig. 22. Activating pathways of organophosphorus compounds.

(e. g., Dimethoate) groups, (c) O-dealkylation, (d) dechlorination (e. g., Phosphamidon), (e) nitro reduction, and (f) conjugation. The hydrolytic reactions may be catalyzed by a variety of esterases present in virtually all the tissues or by microsomal mixed-function oxidases, whereas the other reactions may be mediated by microsomal mixed-function oxidases, nitro-reductase, glutathione S-transferase or glucuronyl transferase. These pathways can evidently transform alkylating agents into nonalkylating metabolites. The metabolism of several representative organophosphorus compounds is briefly discussed below.

Trimethylphosphate is almost inactive as a phosphorylating agent. Many of the esterase-catalyzed reactions do not appear to operate on TMP. The only major metabolic pathway is O-demethylation catalyzed by glutathione-dependent S-alkyl transferase present in the liver. The major urinary metabolites of TMP in rodents are dimethylphosphate (which is inactive as an alkylating agent), S-methylcysteine and its N-acetate (which are derived from S-methyl glutathione) (80, 81). It has been postulated that high doses of TMP are required to saturate this pathway before it may exert deleterious effects (3).

← Fig. 23

The metabolic pathways of Parathion, shown in Fig. 23, are probably representative of many phosphothionate insecticides. Oxidative desulfuration, catalyzed by microsomal mixed-function oxidase, is the principal activation-pathway (82-88). The desulfuration of Parathion (i) is postulated to proceed via a cyclic intermediate, formed by direct addition of atomic oxygen at the P=S bond (88). The resulting metabolite, Paraoxon (ii), is more toxic and more reactive both as phosphorylating and alkylating agent, than the parent

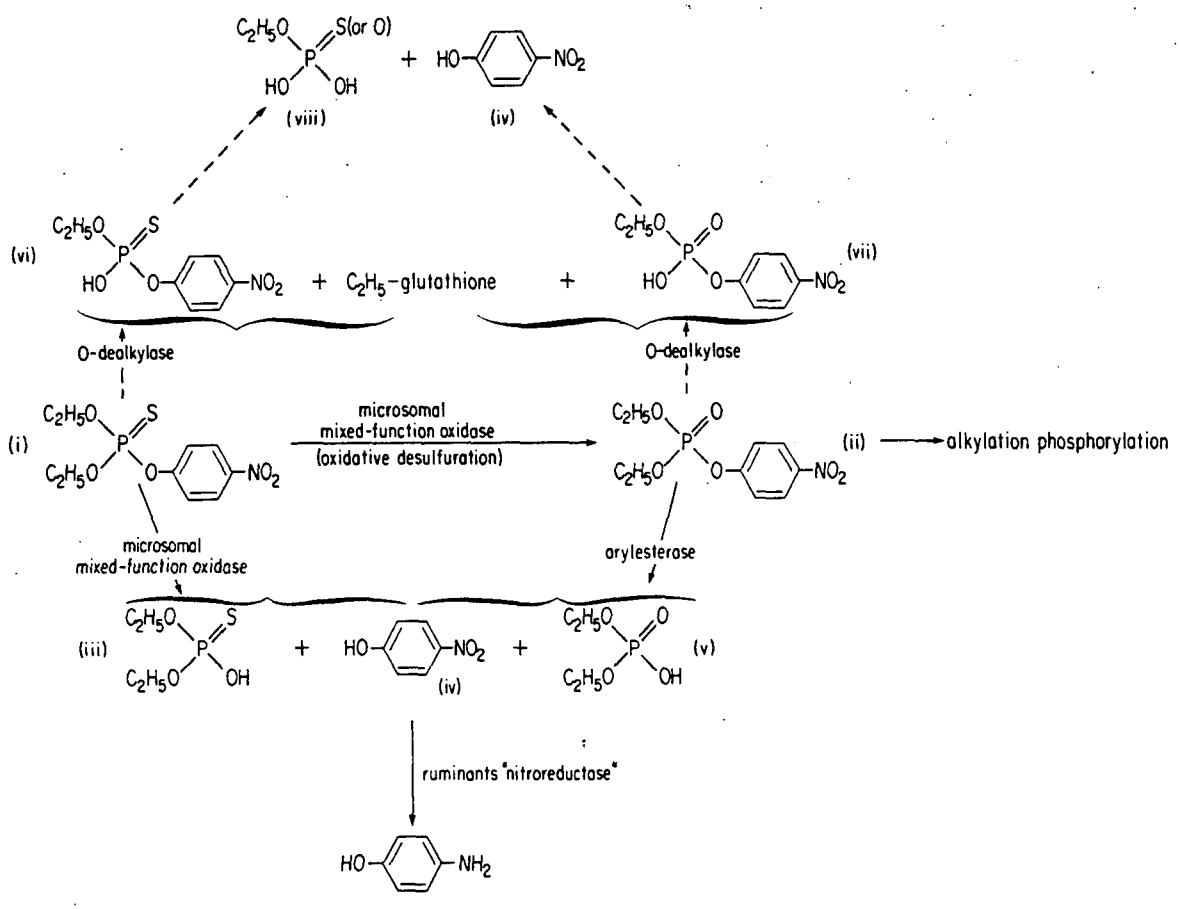

(viii)
(iv)
(vi)
(vii)
+ C_2H_5-glutathione
O-dealkylase
O-dealkylase
(i)
microsomal
mixed-function oxidase
(oxidative desulfuration)
(ii)
alkylation phosphorylation
microsomal
mixed-function oxidase
arylesterase
(iii)
(iv)
(v)
ruminants "nitroreductase"

Figure 23

LEGEND TO FIGURE 23

Fig. 23. Proposed metabolic pathways of Parathion. The chemical names of the compounds are: *i* = Parathion; *ii* = Paraoxon; *iii* = diethylphosphorothionic acid; *iv* = p-nitrophenol; *v* = diethylphosphoric acid; *vi* = desethylparathion; *vii* = desethylparaoxon; *viii* = monoethylthiophosphoric(phosphoric) acid. [Adapted from A. deBruin, "Biochemical Toxicology of Environmental Agents". Elsevies, Amsterdam, 1976, p. 171]

compound. On the other hand, Parathion is also detoxified by microsomal mixed-function oxidase by hydrolytic degradation into diethylphosphorothionic acid (iii) and p-nitrophenol (iv) (88-92). Dearylation is also postulated to proceed via a cyclic intermediate (88), although the microsomal mixed-function oxidase system involved appears to behave differently from that involved in oxidative desulfuration (88, 93, 94). The dearylation of Parathion may also proceed via glutathion-S-aryltransferase present in mammalian liver, degrading Parathion into S-p-nitrophenylglutathione and diethylphosphorothionic acid (95). The dearylation of Paraoxon also proceeds via nonspecific arylesterase(s) present in blood serum and several tissues (96, 97). Another detoxification pathway, although occurring only to a minor extent for both Parathion and Paraoxon, is a glutathione-S-alkyl-transferase catalyzed O-dealkylation reaction. This enzyme is specifically present in the liver and requires NADPH and oxygen for activity. It deethylates Parathion and Paraoxon to desethylparathion (vi) and desethylparaoxon (vii), which eventually give rise to monoethylphosphorothionic acid and monoethylphosphoric acid, respectively (98, 99).

Dichlorvos undergoes very rapid metabolic changes in various mammalian tissues with the concomitant loss of alkylating as well as phosphorylating activity (rev., 4). Blair et al. (100) reported that Dichlorvos could not be detected in any tissues of rodents after exposure to atmospheres containing up to 0.5 μg/l of the compound. Measurable amounts could be detected in several tissues (with the highest in the kidney) after exposure to a high concentration of 90 μg/l. Under these conditions, the half-life of the compound in the kidney was not more than 13.5 min (100). Rapid disappearance of Dichlorvos

was also observed in pigs receiving the compound as vapor via tracheal cannulae (101). As shown in Fig. 24, the metabolism of Dichlorvos (i) proceeds via two major routes, hydrolysis and demethylation. These pathways appear to be common to rodents (102), pigs (101, 103), hamsters and humans (104), although their relative importance shows variations according to the species and route of administration. Hydrolysis is a prominent degradative route in the metabolism of Dichlorvos. The reaction is catalyzed by nonspecific esterases (also variously named hydroxylases or phosphatases) present in nearly all mammalian tissues tested (see ref. 4 for overview). The resultant products are dimethylphosphate (ii), which is excreted unchanged and 2,2-dichloroacetaldehyde (iii), which may either (a) be reduced to 2,2-dichloroethanol (iv) and then conjugated as a glucuronide, or (b) enter the 2-carbon pool and be oxidized to CO_2, transformed to urea, or incorporated into hippuric acid or protein. The demethylation may proceed either by (a) glutathione-dependent S-alkyl transferase-catalyzed transfer of a methyl group to yield S-methyl-glutathione and desmethyldichlorvos (v), or (b) microsomal mixed-function oxidase--catalyzed oxidation of a methyl group yielding desmethyldichlorvos (v) and formaldehyde (vii). In each case, desmethyldichlorvos is further hydrolyzed to monomethylphosphate (vi), phosphate and methanol. The S-alkyl transferase is a soluble liver enzyme (105-109) demonstrated experimentally to function in vivo (4) and its existence is further supported by the finding that depletion of glutathione enhances the acute toxicity of Dichlorvos (78). The oxidative demethylation of Dichlorvos has been shown to occur in vitro (106); but its in vivo role is still undefined.

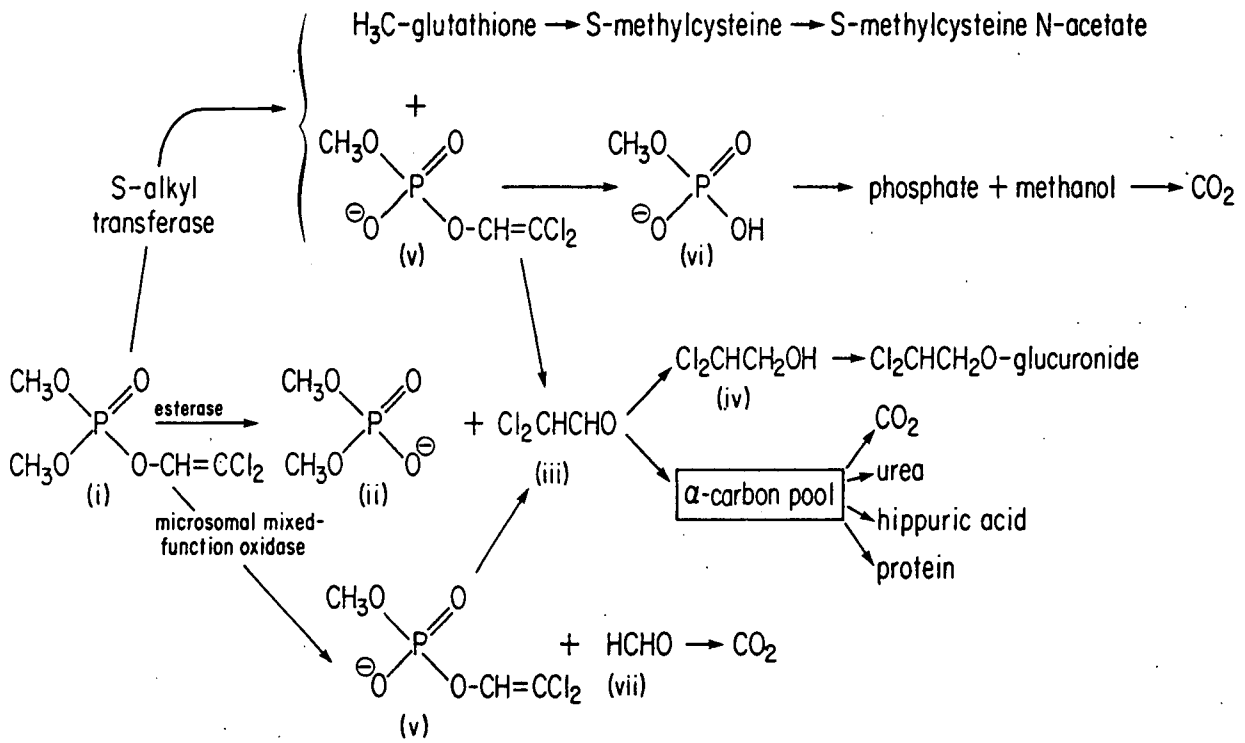

H3C-glutathione → S-methylcysteine → S-methylcysteine N-acetate
S-alkyl transferase
(v)
(vi)
phosphate + methanol → CO2
esterase
(i)
(ii)
+ Cl2CHCHO
(iii)
Cl2CHCH2OH → Cl2CHCH2O-glucuronide
(iv)
α-carbon pool
CO2
urea
hippuric acid
protein
microsomal mixed-function oxidase
+ HCHO → CO2
(vii)

Figure 24

LEGEND TO FIGURE 24

Fig. 24. Proposed metabolic pathways of Dichlorvos. The chemical names of the compounds are: *i* = Dichlorvos; *ii* = dimethylphosphate; *iii* = 2,2-dichloroacetaldehyde; *iv* = 2,2-dichloroethanol; *v* = desmethyldichlorvos; *vi* = monomethylphosphate; *vii* = formaldehyde.

Hexamethylphosphoramide (HMPA) has been shown to undergo sequential demethylation *in vivo* to yield N', N'', N'''-trimethylphosphoramide in rats and mice (110). The N-demethylation also proceeds *in vitro* resulting in the release of formaldehyde using liver slices (110) or 9,000 g postmitochondrial supernatant of liver homogenate (S-9 fraction) (36) as the enzyme source The overall reaction is thought to be detoxifying because the final metabolite lost its biological activity (anti-spermatogenic effect) (110) Even the loss of one methyl group may be inactivating as suggested by the lack of chemosterilant activity of pentamethylphosphoramide in the housefly (111) However, in the process of demethylation, unstable reactive intermediates are produced These intermediates, which may be N-oxide $[(CH_3)_2\overset{\overset{O}{\uparrow}}{N}-]$ or methylol ($CH_3-\underset{|}{N}-CH_2OH$), have been suggested to be the activated form(s) of phosphoramide (110, 112, 113) It has been shown that the cell-transforming activity of HMPA requires metabolic activation by S-9 liver fraction (36, 37)

The metabolic fate of Trichlorfon has not been thoroughly investigated Trichlorfon is readily transformed nonenzymatically into Dichlorvos (2) which may be the actual toxicant. However, this pathway does not seem to operate to any great extent in some species because the hydrolysis of Trichlorfon itself may be faster than that of Dichlorvos in these species (114) After injection of Trichlorfon into dogs, trichloroethyl glucuronide was detected, suggesting the cleavage of the P–C bonding of Trichlorfon In the rabbit, however, a glucuronide of desmethyldichlorvos was found instead, suggesting that the metabolic pathway involved transformation of Trichlorfon to Dichlorvos, followed by demethylation and glucuronidation (115)

Information on the role of metabolism in the activation of Tris(2, 3-dibromopropyl)phosphate (Tris-BP) is not available at the time of writing. As previously discussed, the compound is mutagenic as such; however, inclusion of S-9 liver fraction substantially enhances its mutagenic activity.

The mechanism of carcinogenic action of organophosphorus compounds has not been thoroughly investigated. It is generally assumed that they initiate carcinogenesis by alkylating key cellular macromolecules, although experimental evidence in support of the hypothesis has yet to be obtained. Dichlorvos (which is not considered carcinogenic) is the only organophosphorus insecticide whose reaction with macromolecules has been studied. Lofroth (116) detected first the presence of 7-methylguanine from calf thymus DNA after incubation with Dichlorvos. This *in vitro* reaction has been confirmed by Lawley *et al.* (117) using salmon sperm DNA and *E. coli* DNA. In addition, other minor DNA-methylation products including 1-methyladenine, 3-methyl- adenine, guanine and cytosine, and O^6-methylguanine have been found. ^{14}C-Labeled 7-methylguanine could be detected in the urine of mice given labeled Dichlorvos by i. p. injection (19-39 mg/kg) or inhalation (5 mg/kg), indicating *in vivo* alkylation. However, the possibility that the label arose *via* the 1-carbon pool could not be eliminated. A more recent study by Wooder *et al.* (118) could not demonstrate methylation of nucleic acids in various tissues after exposure of CFE strain rats to atmospheres containing practical use concentrations of Dichlorvos. It has been suggested that the rapid metabolism of Dichlorvos renders the rate of *in vivo* alkylation insignificant.

5.2.1.4.1.4 Environmental Significance. Since organophosphorus compounds are extensively used, human exposure may occur under occupational settings as well as in the general population consuming products containing these compounds as residues.

Trimethylphosphate is used mainly as a methylating agent in the synthesis of organophosphorus insecticides, as a gasoline additive for controlling surface ignition and spark plug fouling, and as a catalyst in the manufacture of polyesters (119). The annual production of the compound in the United States has been estimated to be not more than 1,000 lb in the recent years (120). There is no information available on the extent of human exposure and potential health hazard.

Organophosphorus compounds and chlorinated hydrocarbons are the two most important classes of insecticides used in the United States. Compared to chlorinated hydrocarbons, organophosphorus insecticides are much less persistent and are very unlikely to accumulate in the environment up to toxic levels. The estimated (47, 120-122) figures of annual consumption or production of some of the organophosphorus compounds covered in this subsection are as follows (in millions of lbs. with the year estimated in parenthesis): Methyl Parathion 51 (1972), Malathion 35 (1971), Parathion 15.3 (1970), Dichlorvos 2.7 (1974), and Phosphamidon 1.5 (1974).. The National Institute of Occupational Safety and Health has estimated that about 150,000, 75,000 and 250,000 U.S. workers are potentially exposed to Methyl Parathion, Malathion and Parathion, respectively (47, 121, 122). The literature on the effect of human exposure to these three pesticides has been thoroughly reviewed (47, 121,

122). There is no epidemiologic evidence to suggest any potential carcinogenic risk of human exposure to occupational levels of organophosphorus insecticides. The Time-Weighted Average Threshold Limit Values (TLV-TWA) adopted by the American Conference of Governmental and Industrial Hygienists for air exposure are as follows (in mg compound/m^3 air): Diazinon 0.1, Dichlorvos 1.0, Malathion 10, Methyl Parathion 0.2, and Parathion 0.1 (123).

A variety of organophosphorus compounds have been used as flame retardants in the textile and plastics industries. Among these, Tris-BP is no doubt the most widely publicized compound. An estimated 10 million pounds of Tris-BP was produced in 1976 (120), about 65% of which was applied to polyester and polyacetate fabrics (used for manufacturing children's sleepwear) in compliance of the U.S. Flammable Fabric Act. The remainder was used in other materials, such as acrylic fibers and urethane foams. However, the recent findings of (a) potent mutagenicity, (b) animal carcinogenicity, (c) leaching from the fabric (124), and (d) skin absorption (125) of Tris-BP have prompted a reexamination of the safety of the flame retardant. The problem is further stressed by the fact that children (as all young mammals) are, generally speaking, more susceptible to chemical carcinogenesis than are adults. In early 1977, in view of the potential risk and the nature of the population at risk, the Consumer Product Safety Commission decided to ban the sale of fabrics containing Tris-BP (74). Tetrakis(hydroxymethyl)phosphonium chloride (THPC) is another flame retardant that may represent a potential carcinogenic risk. THPC may break down to its ingredients, phosphine, formaldehyde and hydrochloric acid, and the latter two products may in turn combine to form the potent

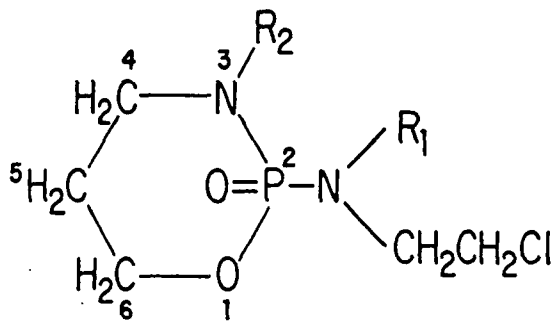
carcinogen, bis(chloromethyl)ether, THPC has now been largely replaced by tetrakis(hydroxymethyl)sulfate (126).

In addition to the above, hexamethylphosphoramide (HMPA) is an effective chemosterilant of the housefly and other insects (127). Also, it has increasingly been used as an industrial solvent in the last few years (66). The annual production of the compound surpassed 1,000 pounds in 1975 (120). However, in view of its very potent inhalational carcinogenicity in rodents, HMPA is regarded to represent a carcinogenic risk for man (123); threshold limit values for the compound have yet to be developed.

5.2.1.4.2 Cyclophosphamide. Cyclophosphamide (Cytoxan, Endoxan, or 2-[Bis(2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide), the structure of which is depicted below, is no doubt the most extensively studied phosphorus-containing alkylating agent. It is a derivative of nitrogen

Insert here Text-Fig. 26

mustard and was originally synthesized in 1958 by Arnold and Bourseaux (161) with the intention of developing a latent cytostatic agent that generates reactive cytotoxic intermediates after activation by the presumed "phosphoridamidase" inside tumor cells. Since the first report (162) of its cytostatic activity, cyclophosphamide has been developed into one of the most important, if not indispensable, cancer chemotherapeutic agents. It has been used, either



Cyclophosphamide $\begin{cases} R_1 = -CH_2CH_2Cl \\ R_2 = -H \end{cases}$

Isophosphamid $\begin{cases} R_1 = -H \\ R_2 = -CH_2CH_2Cl \end{cases}$

Trophosphamide $R_1 = R_2 = -CH_2CH_2Cl$

Text-Figure 26

singly or in combination with other agents, as a cytostatic as well as an immunosuppressive agent, in the treatment of various malignant diseases and nonneoplastic disorders. Interest in the drug is reflected by the enormous number (estimated to be around 10,000 in 1975 by Hill; ref. 163) of publications dealing with the biological properties of the compound. A number of searching reviews on the various aspects of cyclophosphamide have been published (163-166). This section reviews the literature of cyclophosphamide and its newly developed analogs, isophosphamide (iphosphamide, ifosfamide) and trophosphamide (trofosfamide), with special emphasis on carcinogenicity and metabolism.

5.2.1.4.2.1 Physico-Chemical and Biological Properties. The physical and chemical properties of cyclophosphamide have been described (163-166). Cyclophosphamide (monohydrate) is a white crystalline solid with a m. p. of 49.3-53°C. It is soluble in water (40 mg/ml), benzene, chloroform, dioxane and glycols. In unbuffered aqueous solutions, cyclophosphamide slowly hydrolyzes to nornitrogen mustard, phosphate and n-propanol. At temperatures above 30°C, spontaneous hydrolysis of the compound takes place with the liberation of chlorine. Acid-catalyzed hydrolysis of cyclophosphamide gives rise to cytoxylamine. Different other breakdown products are generated under various conditions (rev., 164). Isophosphamide and trophosphamide have properties similar to cyclophosphamide. Isophosphamide is more soluble (130 mg/ml) whereas trophosphamide is less soluble (15 mg/ml) in water than cyclophosphamide (166). In buffered aqueous solution, the relative stability of the three compounds,

as measured by the rate of chlorine liberation, follows the order: trophosphamide > isophosphamide > cyclophosphamide (167, 168).

Cyclophosphamide has a variety of biological activities including toxic, cytostatic, mutagenic and teratogenic effects. Table CIX summarizes the acute toxicity data. The acute toxicity of the drug in the rat appears to be strongly age-dependent; the LD_{50} by s. c. administration is 35 mg/kg for one-day-old rats but increases to 320 mg/kg for 30-day-old rats (169). Investigation of the perinatal toxicity of the drug in the mouse indicates that the drug may exert toxic effects before the full development of the ability of the animal to metabolize the drug to alkylating intermediate(s) (170). The predominant toxic effects in mice, rats and dogs include leucopenia (171) and marked necrosis of the tubular and pelvic epithelia of the urinary bladder (172-174); there is relatively little damage of the liver (175, 176). A detailed review of the toxicological properties of cyclophosphamide has been presented by Hill (163). Isophosphamide and trophosphamide have toxic properties similar to cyclophosphamide; the LD_{50} values in the rat are 250 and 110 mg/kg by i. v. route (177) and 350 and 210 mg/kg by s. c. route (169) for isophosphamide and trophosphamide, respectively.

Shortly after its introduction as a cytostatic drug, cyclophosphamide was found to induce mutation (recessive lethals) in Drosophila melanogaster (178). Since then, numerous studies have been carried out on the mutagenic effects of the drug in a variety of systems including microorganisms, plants, insects and mammals; a detailed review of its mutagenic effects has been published (166). Cyclophosphamide is not mutagenic per se in microbial systems and the

Table CIX

Acute Toxicity of Cyclophosphamide

Species	Route	LD_{50} (mg/kg)	Reference
Mouse	i.p.	400	Schabel cited in ref. 163
		360	271
Rat	oral	94	171
		190	272
		180	273
	i.p.	175	274
	i.v.	160-196	177, 273, 275
	s.c.	35 (newborn)	169
		320	169
Guinea pig	i.v.	400	273
Rabbit	i.v.	130	273
Dog	i.v.	40	273
	oral	44	171

inclusion of a mammalian metabolic activation system is required for activity. Urinary metabolites from cyclophosphamide-treated rats have been shown to induce gene conversion in Saccharomyces cerevisiae D4 (179). The induction of mutation has also been observed in several strains of bacteria (166, 180, 181) in the host-mediated assay. Direct evidence of the involvement of the hepatic mixed-function oxidase system has been recently provided by Ellenberger and Mohn (182) who demonstrated that cyclophosphamide exerted mutagenic effects toward Escherichia coli 343/113 only after incubation with 9,000 x g supernatant (post-mitochondrial fraction) of mammalian liver homogenates (S-9 mix). The induction of dominant lethal mutation has been demonstrated in various strains of mice of both sexes (32, 183-186); there is some preliminary evidence of the induction of specific locus mutation in germ cells of male mice (187). In addition to the above effects, there are a variety of reports of chromosomal aberrations by cyclophosphamide in cultured human or mammalian cells (rev., 166, 188-190). The mutagenicity of isophosphamide and trophosphamide has also been reviewed (166). In comparative investigations in Drosophila, yeast, E. coli and human peripheral leukocytes, the mutagenic potency follows the general ranking: trophosphamide > isophosphamide > cyclophosphamide. The inclusion of a microsomal activation system is required for all three compounds for the expression of activity.

Cyclophosphamide has been reported to exert teratogenic effects in rats (196-200), mice (201, 202), rabbits (203), and chicks (204). In humans, there is some suggestive but inconclusive evidence that cyclophosphamide therapy during pregnancy may induce teratogenic effect (205-207). In rodents, the fetuses

are most susceptible to the teratogenic effect of the drug during the 10th-15th day of gestation. The principal effects include encephaloceles, malformations of the extremities and cervical lesions in rats, whereas mice respond with a high incidence of malformation of the skeleton.

5.2.1.4.2.2 Carcinogenicity. The carcinogenicity of cyclophosphamide has been extensively tested in mice and rats; the major findings of these studies are summarized in Table CX. ← Table CX In the strains of mice used, the lung appears to be the predominant target organ. Tokuoka (208), Duhig (191) and Shimkin et al. (192) were among the first to study the carcinogenicity of the drug in the mouse. Using strains A and dd mice, Tokuoka (208) observed an apparent increase in the tumor incidence after 30 i.p. administrations at a dose of 5 mg/kg. The tumors mainly developed in the lung of strain A mice and in the lung, liver, testis and mammary gland of strain dd mice; however, because of the relatively high spontaneous tumor incidence in these strains, the increases were not statistically significant. The induction of pulmonary adenomas was also observed in strain A mice after five i.p. injections of 0.02 mg cyclophosphamide (191). A more detailed study was carried out by Shimkin et al. (192) using strain A/J mice. Four different dose levels totaling 449, 114, 36 and 9 mg/kg were administered intraperitoneally. Increases in pulmonary tumor incidences were observed; the increase was statistically significant at the second highest dose level. Compared to uracil mustard on a molar basis, cyclophosphamide is about 380 times less potent as a pulmonary carcinogen in the mouse (192). More recently, Kelly et al. (193) have evaluated the carcinogenicity of cyclophosphamide in newborn Charles River CD-1 mice. Intraperitoneal

Table CX

Carcinogenicity of Cyclophosphamide in Rodents

Species and strain	Route	Principal organs affected	Reference
Mouse, A or A/J	i.p.	Lung	191, 192, 208
Mouse, dd	i.p.	Lung, liver, testis, mammary gland (not statistically significant)	208
Mouse, Charles River CD-1 (newborn)	i.p.	Lung (weak)	193
Mouse, Swiss-Webster	i.p.	Lung, bladder	194
Mouse, XVII/Bln or AWD	oral	Lung, liver, skin	195
Mouse, NMRI	s.c.	Mammary gland, ovary, lung, local	209
Mouse, NZB/NZW	s.c.	Lymphoreticular system, local, lung	210-212
Rat, BR46	i.v.	Various sites	209, 213
Rat, Sprague-Dawley	i.v.	Various sites	214
	i.p.	Mammary gland	194
	oral	Bladder, leukemia	276

administration of the drug (0.8, 4.0 or 20.0 mg/kg) within 24 hours of birth and at 3 and 6 days of age elicited only slight increases in pulmonary tumor incidence. More definite evidence of the carcinogenic effect of cyclophosphamide has been provided by Weisburger et al. (194) using Swiss-Webster mice. Intraperitoneal administration of the drug (3x/week, for 6 months; 12-25 mg/kg/dose) led to the induction of lung tumors in 7/30 male and 10/35 female mice. Four male mice also had papillomas of the urinary bladder. The results are statistically significant with $p<0.05$ (194). Carcinogenic effects of cyclophosphamide have also been demonstrated by oral administration of therapeutic doses of the drug. Doses of 0.1 mg/kg/day in the drinking water (up to a total dose of 55 mg) to XVII/Bln and AWD mice led to significant increases in the incidences of predominantly lung tumors, and to a lesser extent, hepatomas and skin tumors (195). The carcinogenicity of cyclophosphamide has also been tested by s.c. administration. Marked carcinogenic effects were demonstrated in female NMRI mice after 52 weekly doses of 26 mg/kg (209). Over 60% (28/46) of the animals developed tumors. The mammary gland was the most affected site with 13 tumors (12 carcinomas and 1 benign); however, tumors also developed in the ovary, lung and at the injection site. For comparison, only 3/46 control mice developed tumors (all stem-cell leukemia) (209). Also in NZB/NZW hybrid mice carcinogenic effects were observed after daily s.c. administration of 1 or 8 mg/kg cyclophosphamide for up to 93 weeks. The lymphoreticular system was the most affected tissue whereas local and pulmonary tumors were occasionally observed (210-212).

Two strains have been used to test the carcinogenicity of cyclophosphamide in the rat. In male BR46 rats, an increase in tumor incidence was first reported by Schmähl (213) after injecting intravenously 50 weekly doses (15 mg/kg) of the drug. Some 54% (14/26) of the treated animals developed tumors (9 malignant and 5 benign) compared to only one benign tumor in 50 control rats ($p < 0.001$). The carcinogenicity of the drug was confirmed in two additional experiments (209). Male BR46 rats receiving 52 weekly i. v. doses of 13 mg/kg had incidences of 11% benign and 17% malignant tumor (compared to 5% benign, 6% malignant in controls); rats receiving 5 biweekly doses of 33 mg/kg had incidences of 8% benign and 24% malignant tumors. There were no distinct target tissues; the spectrum of tumors were: pheochromocytoma, meningioma, sarcoma of the sternum and inguinal region, hemangioendothelioma of abdominal cavity, subcutaneous fibromas and lymphoangioma (209). In Sprague-Dawley rats, the carcinogenicity of cyclophosphamide has been established in three separate expe iments, and it appears that the organotropism of the drug is dependent on the route of administration. By [illegible] i. v. route (weekly injections of 13 mg/kg), Schmähl (214) observed a significant increase in the tumor incidence (14/32 in treated rats compared to 6/52 in controls). Again, there were no distinct target organs; the tumors induced ranged from hemangioendotheliomas in various organs to neurogenic sarcoma of the mediastinum, sarcoma of the heart, leukemia, pheochromocytoma, osteosarcoma of the paranasal sinus, and angiosarcoma of the abdomen. In contrast to the above study, by i. p. route (3 times/week for 6 months, 5-10 mg/kg/dose), the mammary gland was probably the only target organ of cyclophosphamide in Sprague-Dawley-derived

Charles River CD rats. Among the 53 treated female rats, 33 had breast tumors including 9 carcinomas. However, among male rats only 1/50 had a breast carcinoma (194). In a 2-year study, Sprague-Dawley rats receiving daily doses of cyclophosphamide (2.5, 1.25, 0.63, and 0.31 mg/kg) in drinking water had malignant tumor incidences of 19/51, 20/53, 15/28 and 7/13, respectively (compared to 3/24 in controls). Among the cyclophosphamide-treated, tumor-bearing rats, interstitial cell carcinomas of the urinary bladder were found in 8 rats; 5 rats had papillomas of the bladder and 8 had leukemia. These findings support the evidence emerging from case reports that patients treated with cyclophosphamide have an increased risk of bladder tumor and leukemia (see further in "Environmental Significance").

The transplacental carcinogenicity of cyclophosphamide has not been adequately studied. Working with mice, Roschlau and Justus (195) reported that the offspring of mothers treated with cyclophosphamide during pregnancy have a higher incidence of tumors than the offspring from untreated mothers. However, the statistical significance of the study could not be evaluated. Cyclophosphamide has also been shown to bring about, following microsomal activation, in vitro transformation of C3H/10T½CL8 cells (190). The transformed cells could be scored as transformation foci on tissue culture plates and shown to produce sarcomas when injected into syngenic hosts (190).

In contrast to cyclophosphamide, there are very few studies of the carcinogenicity of isophosphamide. Using strain A mice, Stoner et al. (215) found isophosphamide to be a relatively weak pulmonary carcinogen. The total i.p. dose found to elicit significant carcinogenic response ranged from 0.45 to 1.3

g/kg. On the molar basis, isophosphamide is about 200 times less potent than uracil mustard (215). A more detailed study has recently been carried out under the Carcinogenesis Testing Program of the U.S. National Cancer Institute. Sprague-Dawley rats and $B6C3F_1$ mice were used. Isophosphamide could not be clearly shown to induce tumors in male animals of either species. In female animals, however, cancer of the uterus (leiomyosarcoma) as well as mammary fibroadenoma occurred with high incidence in isophosphamide-treated rats whereas mice responded with a high incidence of malignant lymphoma of the hematopoietic system (216). There is no information on the carcinogenicity of trophosphamide.

5.2.1.4.2.3. Metabolism and Possible Mechanism of Action. Cyclophosphamide was originally designed as a latent cytotoxic agent to be enzymatically activated *in vivo* to alkylating intermediates, ideally inside the neoplastic tissues (161). Metabolic studies carried out in a wide range of animal species indicate that the liver is probably the only site where activation of the drug occurs to a significant extent. The metabolic fate of cyclophosphamide in animals has been thoroughly reviewed in 1974 by Torkelson *et al.* (164); since then, new findings have been reported. Figure 25 summarizes the proposed metabolic pathways leading to the formation of major metabolites. The first step of metabolism is assumed to involve mixed-function oxidase catalyzed hydroxylation on carbon-4 of the ring. The 4-hydroxy-cyclophosphamide thus formed is very unstable and readily tautomerizes to its open-chain aldehyde form, aldophosphamide [2-formylethyl N, N-bis(2-chloroethyl)phosphorodiamidate]. Aldophosphamide may, in turn, either be further oxidized by aldehyde oxidase to the

Fig. 25

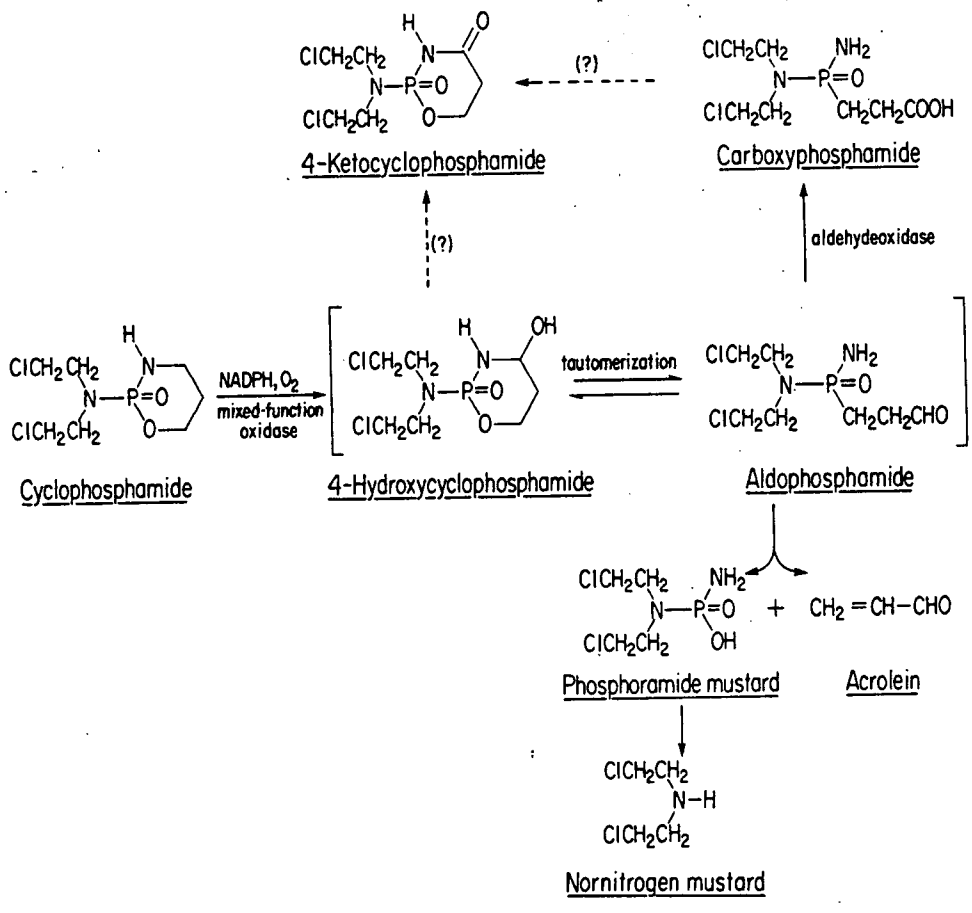

4-Ketocyclophosphamide
(?)
Carboxyphosphamide
(?)
aldehydeoxidase
Cyclophosphamide
NADPH, O2
mixed-function oxidase
4-Hydroxycyclophosphamide
tautomerization
Aldophosphamide
Phosphoramide mustard
Acrolein
Nornitrogen mustard

Figure 25

LEGEND TO FIGURE 25

Fig. 25. Major metabolic pathways of Cyclophosphamide.

carboxyphosphamide [2-carboxyethyl N, N-bis(2-chloroethyl)phosphorodiamidate] or be degraded, either enzymatically or chemically, to phosphoramide mustard [N, N-bis(2-chloroethyl)phosphorodiamidic acid], acrolein and nornitrogen mustard. 4-Ketocyclophosphamide, another urinary metabolite, may arise either by direct oxidation of 4-hydroxycyclophosphamide or by cyclization of carboxyphosphamide.

The involvement of microsomal mixed-function oxidases in the metabolism of cyclophosphamide has been amply demonstrated both by in vivo and in vitro studies. In vivo studies by various investigators (217-221) showed that pretreatment of animals with phenobarbital (a typical inducer) enhances while typical inhibitors, such as 2-diethylaminoethyl 2, 2-diphenylvalerate (SKF 525A) and 2, 4-dichloro-6-phenyl-phenoxyethyl diethylamine (DPEA), inhibit the metabolic activation of cyclophosphamide. In vitro studies indicated that the enzyme system capable of metabolizing cyclophosphamide is located almost exclusively in the microsomes, that it requires NADPH and oxygen for activity, and is inhibited by carbon monoxide, by inhibitors of microsomal electron transport chain and by typical substrates of mixed-function oxidases; it is enhanced by phenobarbital pretreatment (217, 222-225).

Tissue distribution studies showed that only the liver and, to a much lesser extent, the lung contain cyclophosphamide-activating enzyme activity; other tissues such as kidney, spleen, muscle, brain and intestine, and tumor tissues are devoid of this activity (225). 4-Hydroxycyclophosphamide has been identified as a metabolite in the incubation mixture (226), whereas aldophosphamide has been detected in in vitro systems as well as in the plasma and urine

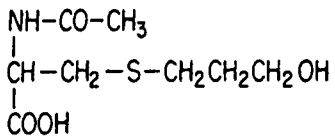
of rats (225, 227). The involvement of aldehyde oxidase in the oxidation of aldophosphamide to carboxyphosphamide has been clearly demonstrated. The conversion is catalyzed by an enzyme present in the cytosol (225, 227); the enzyme can be replaced by purified preparation of aldehyde oxidase (225). Carboxyphosphamide has been detected in the plasma and identified as the principal urinary metabolite in various animal species (227-233) and in humans (229). 4-Ketocyclophosphamide has been found in in vitro systems (234) and as a minor metabolite in the urine of dogs and humans (228, 229, 235). It could arise by oxidation of 4-hydroxycyclophosphamide or by cyclization of carboxyphosphamide. However, the latter pathway is not supported by the study of Takamizawa et al. (230) who were unable to detect any interconversion between carboxyphosphamide and 4-ketocyclophosphamide in the rabbit. Phosphoramide mustard and acrolein have been postulated to arise from the degradation of aldophosphamide although the nature of the degradation is not clear. Phosphoramide mustard can be detected as an in vitro metabolite (226, 236) as well as in the plasma of patients receiving cyclophosphamide (237). Further degradation of phosphoramide mustard yields nornitrogen mustard, which has been identified as an urinary metabolite (238, 239). Acrolein is formed in in vitro metabolism or in chemical oxidation of cyclophosphamide (233, 240). Kaye and Young (241) have provided indirect evidence of acrolein as a possible metabolite of cyclophosphamide in humans. In fact, 3-hydroxypropylmercapturic acid (see Fig. 26) a known metabolite of acrolein in the rat (242), has been detected in the urine of patients receiving the drug.

← Fig. 26

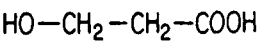
In addition to the above metabolites, a variety of minor metabolites (Fig. 26) have been identified in the urine of various animal species. These are 3-hydroxypropionic acid and 3-hydroxypropionamide in the dog (229), 2-[(2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide and 3-phosphorylpropionic acid in the sheep (232), and 3-(2-chloroethyl)-oxazolidone and 1,4-di-(2-chloroethyl)piperazine in humans (239). There is also some evidence that N-(2-chloroethyl)-ethyleneimine is a minor metabolite excreted in the expired air in very small amounts by rats (243). A number of breakdown products could arise as a result of hydrolysis of cyclophosphamide (rev., 164), and some of these may be of biological significance. Cytoxyl amine, for example, has been detected in the serum of rats and urine of humans given the parent compound (244).

The metabolic fate of isophosphamide closely resembles that of cyclophosphamide. Metabolites corresponding to those of cyclophosphamide (including acrolein) have been detected (226, 245-248). Trophosphamide is expected to be metabolized in a similar manner (166, 249).

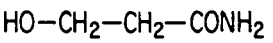
The main thrust of the metabolic studies of cyclophosphamide has been to identify active intermediates accounting for the cytostatic activity of the drug. Relatively little effort has been devoted to identify carcinogenic intermediates. However, in view of the often-observed ambivalence of cytostatic therapy, it is likely that a similar mechanism may operate. A recent study by Benedict et al. (190) indicates that the in vitro oncogenic transformation of C3H/10T½CL8 cells by cyclophosphamide requires microsomal mixed-function oxidases, NADPH and oxygen for activation.



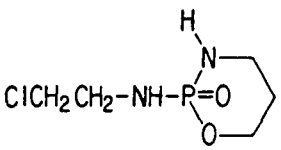
3-Hydroxypropylmercapturic acid



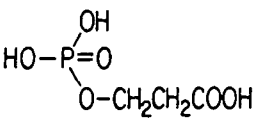
3-Hydroxypropionic acid



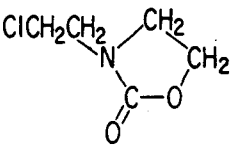
3-Hydroxypropionamide



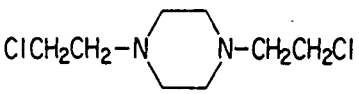
2-[(2-chloroethyl)amino]-tetrahydro-2H-1,2,3-oxazaphosphorine 2-oxide



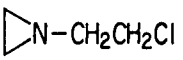
3-Phosphorylpropionic acid



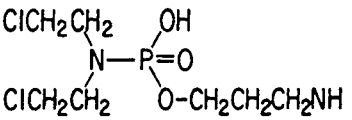
3-(2-chloroethyl)-oxazolidone



1,4-Di-(2-chloroethyl) piperazine



N-(2-chloroethyl)ethyleneimine



Cytoxyl amine

Figure 26

LEGEND TO FIGURE 26

Fig. 26. Some minor metabolites of Cyclophosphamide.

It appears to be generally accepted that the two principal urinary metabolites, carboxyphosphamide and 4-ketocyclophosphamide are "detoxified" metabolites, and do not represent active forms of the drug (225, 229, 234). The intermediates postulated to be possible active cytostatic intermediates include 4-hydroxycyclophosphamide, aldophosphamide, phosphoramide mustard, and nornitrogen mustard. Although acrolein is believed by some (233) to be related to the therapeutic effect of cyclophosphamide, others (250) consider that it is unlikely to play a major role in the cytostatic action but may contribute to some of the toxic effects of the drug. The potential carcinogenicity of acrolein will be discussed in Section 5.2.1.7.

The alkylating properties of potentially active intermediates have been studied. Sladek (227) presented evidence that an aldehyde with alkylating activity is the primary *in vitro* metabolite of cyclophosphamide as well as the primary *in vivo* metabolite in the blood and urine of treated rats. The aldehyde is capable of alkylating 4-(*p*-nitrobenzyl)pyridine, can be trapped as semicarbazone by the addition of semicarbazide, and was concluded to be aldophosphamide. The ability of 4-hydroxycyclophosphamide, phosphoramide mustard and nornitrogen mustard to alkylate 4-(*p*-nitrobenzyl)pyridine has been compared by Struck *et al.* (251). Under the conditions used, 4-hydroxycyclophosphamide was inactive; both phosphoramide mustard and nornitrogen mustard were active alkylators at acidic pH but only phosphoramide mustard retained activity at physiological pH. The authors (251) postulated that phosphoramide mustard is physiologically the most significant cytostatic intermediate generated by cyclophosphamide metabolism. This conclusion is strongly supported

by the finding of Colvin et al. (250) who confirmed the potent alkylating ability of phosphoramide mustard at physiological pH and showed in addition, by mass spectrometry, the binding of the whole molecule (not its degradative product, nornitrogen mustard) to the nucleophile, ethanethiol. The postulated reactive intermediates are depicted in Fig. 27. It should be noted, however, that phosphoramide mustard alone does not account for all the cytostatic effects of cyclophosphamide (252); Colven et al. (250) pointed out that the assay techniques used by Struck et al. (251) could not preclude the existence of low but biologically significant levels of alkylation, and implied that either 4-hydroxycyclophosphamide is the key metabolite to enter target cells or that other additional factors are involved. Further studies are needed to elucidate whether any of these alkylating intermediates or some other minor metabolites are related to the carcinogenic effect of the drug.

5.2.1.4.2.4 Environmental Significance. Human exposure to cyclophosphamide is probably limited exclusively to iatrogenic sources. Cyclophosphamide is one of the most extensively used chemotherapeutic agents in the treatment of various malignancies such as myeloma, leukemia, Hodgkin's disease, and ovarian, mammary and pulmonary tumors. It has also been used in combination with other drugs in the therapy of lymphoreticular and a variety of other neoplasms (165, 253, 254). In addition to its antitumor activity, cyclophosphamide is an effective immunosuppressive agent and is increasingly used after organ transplantation (255). Other non-neoplastic diseases for which cyclophosphamide has been used or clinically tested is the treatment of rheumatoid arthritis (256),

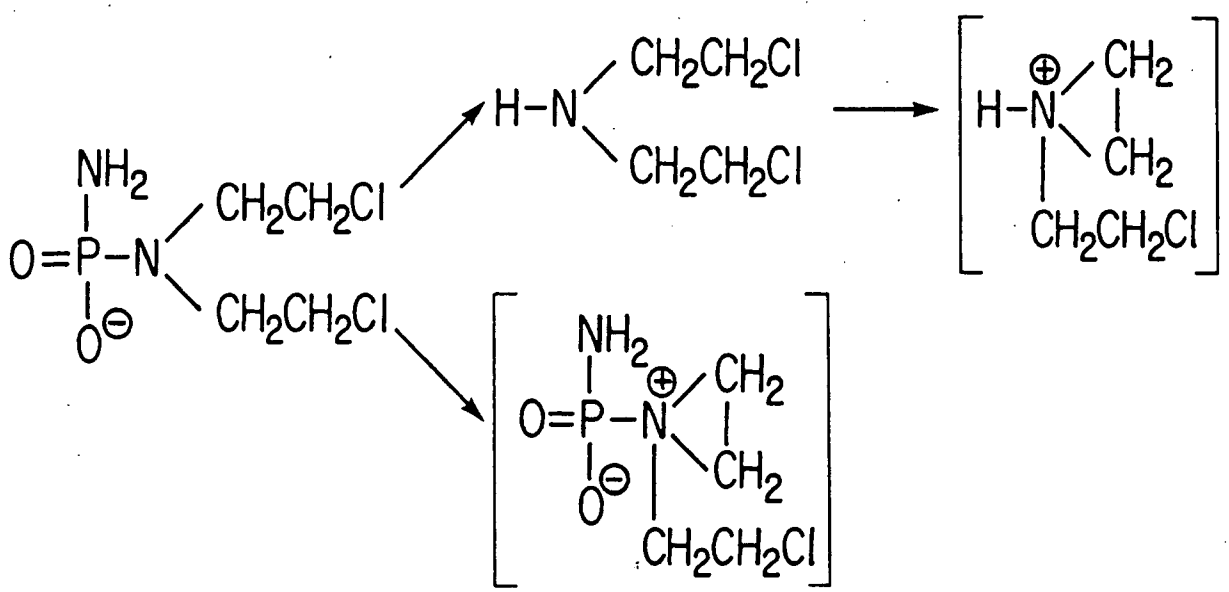

NH2
O=P-N
O⊖
CH2CH2Cl
CH2CH2Cl
H-N
CH2CH2Cl
CH2CH2Cl
H-N⊕
CH2
CH2
CH2CH2Cl
NH2
O=P-N⊕
O⊖
CH2
CH2
CH2CH2Cl

Figure 27

LEGEND TO FIGURE 27

Fig. 27. Possible reactive intermediates formed from phosphoramide mustard.

chronic hepatitis (257), systemic lupus erythematosus (258), idiopathic nephrotic syndrome in children (259) and a number of others (165, 260).

A variety of antineoplastic drugs have been found to possess carcinogenic activity (rev., 261, 262). The potential cancer risk of patients receiving cyclophosphamide therapy has been reviewed and evaluated by IARC (165), and by Schmähl et al. (262). There have been at least 40 cases of malignant tumors reported in patients treated with cyclophosphamide for various diseases, and in nearly half of these cases, cyclophosphamide was the only chemotherapeutic agent administered. There is increasing evidence to suggest that therapeutic use of the drug may present an increased risk of cancer of the urinary bladder. Seven cases of the development of bladder carcinomas as second primary tumors have been reported in cancer patients receiving cyclophosphamide as the sole chemotherapeutic agent (263-265). In addition, seven other cases of bladder tumors have been noted in patients receiving combination chemotherapy in which cyclophosphamide was one of the drugs (264-267; Laursen, cited in ref. 262). Schmähl et al. (262) have pointed out that the reported and published cases may represent only a fraction of the total number of cyclophosphamide-related bladder cancers. Of the cases in which the total applied dose of the drug could be estimated, the total doses were found to range from 100-250 g. The mean latent period for bladder cancer was 7 years (262). In addition to bladder cancer, cyclophosphamide could be related to the development of 3 cases of leukemia (265, 268), and reticulosarcomas (269, 270) in patients receiving the drug for therapy of malignant diseases, and 2 cases of reticulum cell sarcoma and 1 case each of chronic lymphocytic leukemia, Hodgkin's

disease, cervical cancer, malignant melanoma, astrocytoma and glioblastoma in patients treated for nephrotic syndrome and other nonneoplastic disorders (rev., 165). Whether or not these tumors are directly related to the drug treatment could not be assessed because of the insufficiency of data. Nevertheless, the increasing number of reports of possible association between bladder cancer and cyclophosphamide is alarming and suggests that the drug should be regarded as a human carcinogen.

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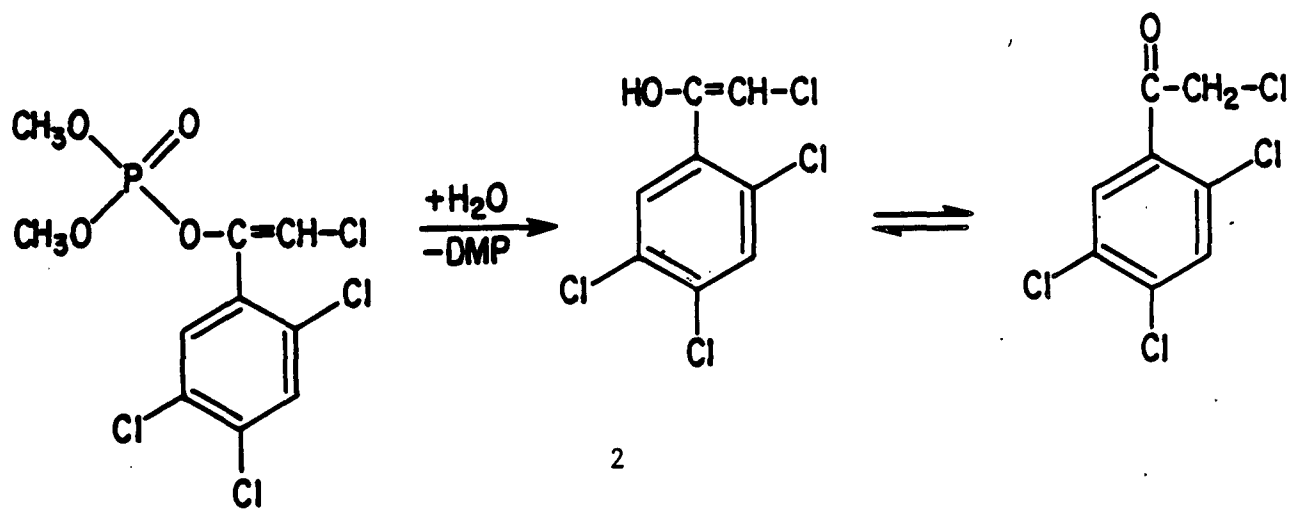
NOTES ADDED AFTER COMPLETION OF SECTION 5.2.1.4

The mutagenicity of 11 bis- or tris-(haloalkyl)-phosphates has been tested by Nakamura et al. (1) using the Ames Salmonella test. Most of the compounds are base-pair substitution mutagens and are more active in the presence of metabolic activation system (S-9 mix). Several structure-activity relationships have been noted: (a) bromoalkyl compounds are more mutagenic than chloroalkyl compounds, (b) haloethyl derivatives are more mutagenic than γ-halopropyl derivatives, (c) compounds with β,γ-dihalo-substitutions are particularly potent, and (d) branching of the alkyl chain reduces the mutagenicity. These relationships do not all apply to haloalkanols suggesting that tris-(haloalkyl)-phosphates are not necessarily activated via haloalkanols. MacGregor et al. (2) have tested the mutagenicity of 9 organophosphorus flame retardants. Only tris-(2,3-dibromopropyl) phosphate was mutagenic. All the other phosphonium, phosphine, phosphine oxide, and phosphoric amide derivatives (none of which contain halogenated side-chains) did not exhibit mutagenic activity. McCoy et al. (3) have recently found that illumination of tris-(2,3-dibromopropyl)-phosphate with visible light in the presence of riboflavin resulted in the formation of a stable intermediate with greatly enhanced mutagenic (Ames test) and DNA-modifying activities. Since illumination of riboflavin is known to generate short-lived singlet oxygen, it is assumed that the genotoxic intermediate results from a reaction between the flame retardant and singlet oxygen. This finding may be of environmental significance because the polluted urban atmosphere is conducive to the generation of singlet oxygen. Two organophosphorus pesticides, dimethoate and phosphamidon (see Figure 20), have been found to be highly mutagenic in the micronucleus test (using mouse bone marrow cells) and in the host-mediated test (using Salmonella typhimurium G46). In the same study, two organo-

chlorine (aldrin and endosulfan), a carbamate (carbaryl) and a mercuric (ceresan) pesticides are not significantly active (4). The mutagenicity studies of Dichlorvos have recently been reviewed by the International Commission for Protection against Environmental Mutagens and Carcinogens (5). A series of derivatives of Diazinon (see Figure 20), pyridyl phosphorothioate and other organophosphorus compounds have been tested for teratogenicity using chick embryos, and some interesting structure-activity relationships have been described. The position, size, and branching of N-heterocyclic ring substituents in the organophosphorus compounds all play a role in determining the potency and the type of teratogenic effects (6).

No new animal carcinogenicity studies have been found in the literature since the completion of Section 5.2.1.4. The evidence of possible carcinogenicity of Dichlorvos has been reviewed by an international study group (5). Two additional cases of transitional cell carcinoma of the bladder have been observed among 54 patients treated with oral doses of cyclophosphamide for systemic lupus erythematosus and rheumatoid arthritis. The tumors were detected 28 and 60 months after the withdrawal of the drug (7).

A possible reaction mechanism for carcinogenesis by tetrachlorvinphos (Rebon) has been pointed out by Burchfield and Storrs (8). Tetrachlorovinphos may be readily hydrolyzed to yield dimethylphosphate (DMP) and a vinyl alcohol, which tautomerizes to yield a highly reactive ω-chloroacetophenone



derivative. The ω-chlorine atom of the intermediate is highly reactive due to its promimity to the electron-rich carbonyl group, which in turn is in resonance with the π electrons of the benzene ring. The intermediate may react with nucleophilic sites in macromolecules to initiate carcinogenesis. The three chlorine atoms on the benzene ring are relatively inactive; they could enhance the carcinogenicity of the compound by altering its physico-chemical properties. The *in vivo* binding of tris-(2,3-dibromopropyl)-phosphate (TRIS) and tris-(1,3-dichloro-2-propyl)-phosphate (Fyrol FR-2) in the mouse has been studied by Morales and Matthews (9). The highest level of binding was the kidney (the main target tissue) for TRIS and the liver for Fyrol FR-2. The covalent binding to DNA was substantially higher for TRIS than that for Fyrol FR-2. The *in vitro* reaction of phosphoramide mustard, a metabolite of cyclophosphamide, with nucleosides (guanosine or deoxyguanosine) has been demonstrated by Mehta *et al*. (10). The major reaction product was phosphoramide mustard with one arm having reacted with the nucleoside at the 7-position. In the DNA, such an adduct is expected to be much less stable than 7-methylguanine and may contribute to cytotoxic, mutagenic or carcinogenic action by causing damage by strand scission.

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