INSECTICIDE METABOLISM IN MAMMALS

Mammalian Enzymes Involved in the Degradation of 2,2-Dichlorovinyl Dimethyl Phosphate

ERNEST HODGSON[1] and JOHN E. CASIDA

Department of Entomology, University of Wisconsin, Madison, Wis.

The metabolic fate of 2,2-dichlorovinyl dimethyl phosphate (DDVP or Vapona) in mammalian tissue homogenates and plasma was investigated. Enzymatic systems active in hydrolyzing DDVP to dimethyl phosphate and methyl 2,2-dichlorovinyl phosphate, and in further hydrolysis of the latter disubstituted phosphate to monomethyl phosphate and finally inorganic phosphate, are reported. The dichlorovinyl portion of the molecule was converted via dichloroacetaldehyde predominantly to dichloroethanol, although trace amounts of dichloroacetic acid also appeared to be formed. Subcellular enzyme localization, metallic ion activation, and the products formed in the reactions studied individually and in sequence are considered.

THE INSECTICIDE 2,2-dichlorovinyl dimethyl phosphate has been extensively tested for use in baits, space sprays, aerosols, and possibly as a fumigant and animal spray. The principal constituent, 2,2-dichlorovinyl dimethyl phosphate, is susceptible to hydrolysis at the vinyl phosphate and *O*-methyl phosphate groupings (*8*, *9*, *25*) and is destroyed by oxidizing agents that attack the vinyl group (*3*). Vinyl phosphate cleavage is catalyzed by hydroxyl ions to form dimethyl phosphoric acid and dichlorovinyl alcohol. The dichlorovinyl alcohol is not stable but is isolated as dichloroacetaldehyde (*16*, *25*). In the presence of alkali the dichloroacetaldehyde may be further decomposed (*9*). Reaction of DDVP and higher dialkyl analogs with esterases presumably yields the dialkyl phosphorylated esterase and dichloroacetaldehyde (*4*, *16*). Mammalian and insect tissues and homogenates active in detoxification of DDVP and related compounds have been extensively investigated (*18*, *26*). No critical attempts have been made to define the total metabolic pathway and enzymatic systems involved in degradation of any of the organophosphorus insecticides. The in vivo biological instability of DDVP is well established based on bioassay and radiotracer studies (*8*, *32*).

The metabolic pathway of DDVP and the enzymes involved in the degradation were investigated using enzyme preparations from mammalian tissues. DDVP was selected as a desirable substrate because of its relatively simple structure, moderate degree of water solubility, and rapid in vivo degradation involving reaction at several sites on the molecule.

Methods and Materials

Chemicals. The DDVP was provided by Shell Development Co. (Modesto, Calif.) as the nonradioactive material for use as an analytical standard, as the P^{32}-tagged material, and with C^{14} in the alpha or 1 position of the dichlorovinyl group. All radioactive preparations were purified by column chromatography on silica gel with pentane-chloroform mixtures (*8*), and infrared spectra of the fractions were examined before use. Methyl 2,2-dichlorovinyl phosphate, designated as the des-methyl derivative, was provided by the Shell Development Co. as the sodium salt, m.p. 227–229° C. (decompd.). This material was prepared by refluxing equimolar DDVP and sodium iodide in acetone, followed by recrystallization from acetone. Phosphorus and chlorine content were appropriate for the proposed structure and ion exchange chromatography showed less than 5% mono- or dimethyl phosphates either contaminating, or formed on subjecting the chemical to the acid chromatographic solvents. Dimethyl phosphate was prepared by chlorination of dimethyl hydrogen phosphite and hydrolysis of the resulting dimethyl phosphoryl chloride. Monomethyl phosphate was purified by crystallization as the barium salt from a commercial mixture of methyl phosphates [procedures reviewed by Kosolapoff (*17*)]. Prior to use as enzyme substrates these methyl phosphates were converted to sodium or potassium salts. Sources for other organic chemicals were as follows: purified dichloroacetaldehyde from Shell Development Co.; dichloroethanol from Sapon Laboratories (Lynbrook, L. I., N. Y.); and the remainder from Eastman Organic Chemicals (Rochester, N. Y.). The following biochemicals were obtained from California Corp. for Biochemical Research (Los Angeles, Calif.); diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), and alcohol dehydrogenase (purified from horse liver, crystallized, ADH-L 15320).

Other organophosphorus compounds used were of the highest purity obtainable from the manufacturers. Chemical designations for the compounds are indicated in Table VII. Emulsions were used for Butonate, Chlorophan, and Dibrom as they were not soluble in the buffer at 0.01*M*. The final concentration of Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) used in preparing these emulsions was 0.5%.

Enzyme preparations. Male albino rats (200 to 250 grams) were sacrificed and the liver was washed with distilled water and placed on ice. All subsequent operations were carried out at 0° to 3° C. Whole homogenates were prepared by homogenization of minced liver in potassium phosphate buffer (0.05*M*, pH 7.0) for 30 seconds using a Lourdes homogenizer with the variable resistor at a setting of 30. Mitochondria were prepared by centrifugation of the whole homogenate at 15,000G for 15 minutes following an initial centrifugation at 1500G for 10 minutes to remove nuclei, whole cells, and debris. The mitochondria were washed three times by resuspending in buffer and recentrifuging at 15,000G for 10 minutes. The soluble fraction and microsomes were prepared by centrifugation of the supernatant from the preparation of mitochondria at 105,000G for 60 minutes. The residue and supernatant were designated microsomes and soluble ($S_{105,000}$), respectively.

Plasma of rats and rabbits was prepared from heparinized blood. Whole homogenates of liver, kidney, and spleen were made in glass homogenizers (*30*) for manometric studies.

Manometric Studies. Hydrolysis of DDVP and some related phosphate esters was followed manometrically using bicarbonate buffer (*18*). Ten milliliter single side-arm Warburg flasks and a 2.0-ml. reaction volume were used. Prior to tipping in the side arm contents, all flasks were gassed with 95%

[1] Present address, Department of Entomology, North Carolina State College, Raleigh, N. C.

nitrogen–5% carbon dioxide for 7 minutes. Enzyme activity in the presence of excess substrate was determined from the initial rate of carbon dioxide evolution at 37° C. The time required for complete substrate hydrolysis was also determined by this procedure. Appropriate controls to correct for nonenzymatic hydrolysis of the substrate and endogenous activity of the enzyme preparations were run with each experiment. The data on the enzymatic hydrolysis of methyl 2,2-dichlorovinyl phosphate have not been corrected for the incomplete ionization of the secondary P—OH group released under the assay conditions. By assuming that monomethyl phosphate is the only acid formed, a pK_a of 6.31 for the secondary hydroxyl of this compound (*20*) can be used for correction.

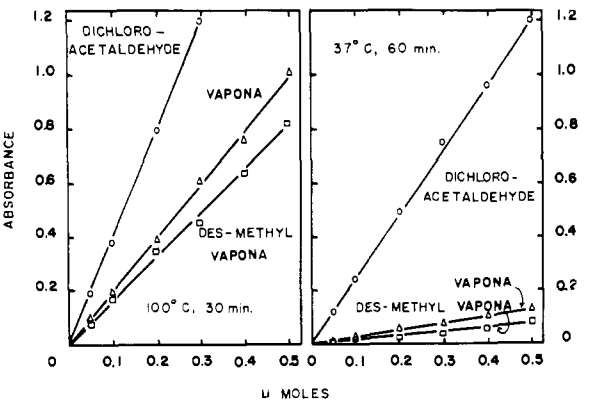

Figure 1. Standard curves for DDVP, des-methyl DDVP, and dichloroacetaldehyde using the 2,4-dinitrophenylhydrazine method and varying reaction conditions

Column Chromatography of Phosphorus-Containing DDVP Metabolites. The phosphorus-containing metabolites from DDVP-P^{32} were separated by ion-exchange chromatography using Dowex 1-X8 in a similar manner to a described procedure (*27*). Elution was achieved with the following series of solvents: 1) gradient from 150 ml., pH 2 to 150 ml., pH 1.4 hydrochloric acid; 2) gradient from 150 ml., pH 1.4 to 150 ml., pH 1 hydrochloric acid; 3) gradient from 200 ml., pH 1 hydrochloric acid plus methanol (1:3) to 200 ml. 1*N* hydrochloric acid plus methanol (1:3); and 4) 200 ml. of concentrated hydrochloric acid, water, and acetone (1:1:6). Eleven milliliter fractions were collected. The position and identity of the eluted compounds were determined by cochromatography with authentic compounds, inorganic phosphate and total phosphorus determination, and radioactive counting. Samples were prepared for chromatography by precipitation of the proteins with an equal volume of acetone followed by removal of the acetone under reduced pressure and the unreacted DDVP by extraction with chloroform.

Chemical Methods. Inorganic and total phosphorus were determined by the method of Allen (*2*). The hydrolysis of monomethyl phosphate was determined from the amount of inorganic phosphate released on incubation with enzyme preparations. For these experiments enzyme preparations were made in tris-(hydroxymethyl)-aminomethane-HCl or tris-HCl buffer (pH 7.4, 0.05*M*). Proteins were precipitated prior to phosphate analysis by adding trichloroacetic acid to a final concentration of 3%.

The observation that dichloroacetaldehyde formed a derivative with 2,4-dinitrophenylhydrazine, which yielded an intense blue color on the addition of alkali, made possible its determination by a modification of the method used for triose phosphates (*7*, *21*). After precipitation and removal of proteins by the addition of trichloroacetic acid until a concentration of 3% was present, a 1.0-ml. aliquot was added to 1.0 ml. of a 0.1% solution of 2,4 - dinitrophenylhydrazine in 2*N* hydrochloric acid and incubated at 37° C. for 60 minutes. Addition of 7.0 ml. of 0.75*N* sodium hydroxide caused the formation of a blue precipitate which dissolved completely on the addition of an equal volume of methyl Cellosolve. The absorbance at the absorption maximum, 590 mμ, was measured in a Bausch and Lomb Spectronic 20 colorimeter. Figure 1 shows standard curves for the color formation from dichloroacetaldehyde, des-methyl DDVP, and DDVP at two different temperatures. By selecting suitable reaction conditions, it was possible to determine dichloroacetaldehyde in the presence of des-methyl DDVP. In the present experiments, the lower reaction temperature (37° C., 1 hour) was used to study the release of dichloroacetaldehyde on enzymatic hydrolysis of des-methyl DDVP. Variation of the reaction conditions might permit use of this method for analysis of DDVP residues. With suitable reaction volumes, as little as 1.0 μg. of DDVP could be determined.

The color developed by certain other organophosphates when reacted as described above at 100° C. for 30 minutes was determined. Chlorophan, Phosdrin, and alpha-3562 gave no color. Dichloroacetaldehyde, DDVP, Butonate, and Dipterex yielded a blue color with an absorption maximum at 590 mμ, the relative intensity of color with equimolar amounts being 1.0: 0.59: 0.38: 0.25. Dibrom yielded a red-brown color which corresponded closely with that from chloral, the absorption maximum being 460 mμ. Examination of the absorption spectra of the colored reaction mixtures using a Beckman DK2 recording spectrophotometer showed the peaks at 590 and 460 mμ to be broad and overlapping.

Radiotracer Procedures. All radioactive samples were counted in a thin window Geiger-Müller counter with a Nuclear-Chicago 64 scaler. Samples containing P^{32} were counted directly in solution by placing 1.0-ml. aliquots in aluminum planchets. On partitioning between chloroform and water DDVP appears completely in the chloroform phase and the hydrolysis products completely in the aqueous phase. The hydrolysis of DDVP-P^{32} was followed by partitioning aliquots of the incubation mixtures, taken at various times, between equal volumes of chloroform and water and counting the radioactivity in the two layers. Dimethyl phosphate-P^{32} was prepared by alkaline hydrolysis of DDVP under identical conditions to those described below for obtaining dichloroacetaldehyde-C^{14} from DDVP-C^{14}.

DDVP-C^{14} was counted in the presence of a volatility depressant or after conversion to a nonvolatile labeled derivative. Arochlor-5460 was used as a volatility depressant (*15*) by adding an equal volume of pentane containing 1.0 mg. of Arochlor to the DDVP in organic solvent in an aluminum planchet. The solvent was allowed to evaporate completely before counting. Alternatively, the DDVP was converted to the 2,4-dinitrophenylosazone of glyoxal as indicated below, and this derivative was counted.

Dichloroacetaldehyde - 1 - C^{14} was prepared from DDVP-C^{14} by complete hydrolysis of the vinyl phosphate bond in 0.2*N* sodium hydroxide at 27° C. for 15 minutes, and the pH was then adjusted to 7.0 with hydrochloric acid. This set of conditions yielded complete vinyl phosphate cleavage based on preliminary studies with DDVP-P^{32}. The C^{14}-labeled aldehyde in enzymatic preparations was counted as follows: 2.0 ml. of 2*N* hydrochloric acid was added to the 5 ml. of incubation mixture, and the protein precipitate was removed by centrifugation. To a suitable aliquot

Table I. Rate of Hydrolysis of DDVP and Des-Methyl DDVP by Homogenates of Tissues of Rat and Rabbit

Tissue	μL. CO_2/Min./G. Tissue: DDVP	μL. CO_2/Min./G. Tissue: Des-Methyl DDVP	Activity Ratio, Des-Methyl DDVP/DDVP
Rat			
Liver	37	67	1.81
Kidney	23	25	1.09
Spleen	10	13	1.30
Plasma	5	0.3	0.06
Rabbit			
Liver	36	42	1.17
Kidney	34	38	1.12
Spleen	17	16	0.94
Plasma	31	0.5	0.02

Table II. Effect of Metal Ions on DDVP Hydrolysis by Rat Liver Homogenates

Added Cation ($1 \times 10^{-3}M$)	μL. CO_2/Min./G. Tissue: Fresh homogenate	Added EDTA-Na_2	Dialyzed against EDTA-Na_2
None	37	37	8
Ca	54	61	18
Co	17	34	10
Mg	30	35	8
Mn	47	67	39
Sr	36	40	9

Table III. Effect of Metal Ions on DDVP Hydrolysis by Subcellular Fractions of Rat Liver

Molarity of Cation	μL. CO_2/Min./G. of Equiv. Fresh Wt.: Ca	Co	Mn
Mitochondria			
None	6	6	6
1×10^{-4}	18	5	6
1×10^{-3}	17	7	4
Soluble ($S_{105,000}$)			
None	31	31	31
1×10^{-4}	38	35	47
1×10^{-3}	31	42	70

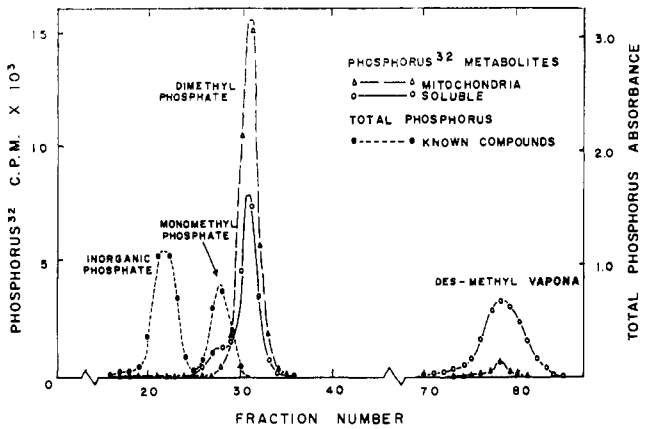

Figure 2. Hydrolysis products from DDVP-P^{32} following incubation with rat liver soluble ($S_{105,000}$) and mitochondrial enzymes

of the supernatant, 1.0 ml. of a solution of dichloroacetaldehyde (1.0 mg./ml. water) and 5.0 ml. of 2,4-dinitrophenylhydrazine (1.0 mg./ml. 2*N* hydrochloric acid) were added, and after heating at 100° C. for 30 minutes, the crystals were filtered off, washed with a large volume of 2*N* hydrochloric acid, dried, and counted. The derivative prepared in the above manner from an authentic sample of dichloroacetaldehyde decomposed at 314–318° C. The published melting point for the 2,4-dinitrophenylosazone of glyoxal formed with the same reactants is 313–314° C. with decomposition (*16*).

Dichloroacetic acid-1-C^{14} was prepared from DDVP-C^{14} by alkaline hydrolysis in the presence of iodine (*9*). DDVP was added to 0.36*N* sodium hydroxide in the presence of 0.045*N* iodine and 0.064*M* sodium iodide. After 10 minutes at 27° C. the reaction mixture was strongly acidified with hydrochloric acid, and the labeled acid was extracted into ether. Dichloroacetic acid-C^{14} from enzymatic preparations was counted as follows. The acidified incubation mixture (see above) was extracted twice with an equal volume of ether; 25 mg. of nonlabeled dichloroacetic acid and 50 mg. of dicyclohexylamine were added in small volumes of ether. The salt which precipitated was filtered off, washed with ether, dried, and counted. An authentic sample of dichloroacetic acid converted to the salt in this manner yielded needles melting at 192–194° C. and decomposing at 204–208° C., while the salt of monochloroacetic acid appeared as plates melting at 154-156° C.

Dichloroethanol-1-C^{14} was prepared enzymatically from dichloroacetaldehyde-1-C^{14} by reaction with 1 μg. of crystallized alcohol dehydrogenase and excess DPNH for 30 minutes at 37° and pH 7 in a 3.0-ml. reaction volume. Dichloroacetaldehyde was completely reduced under these conditons as ascertained by the phenylhydrazine method. Dichloroethanol-C^{14} was converted to the phenyl urethane for counting in the following manner. The incubation mixture was extracted with three successive 3.0-ml. aliquots of benzene; the benzene was dried with anhydrous sodium sulfate and refluxed for 2 hours after adding 100 mg. of nonradioactive dichloroethanol and 500 mg. of phenylisocyanate. On evaporation of the solvent, crystals of the phenyl urethane of dichloroethanol separated and were recrystallized two to four times from hexane, dried, and counted. The phenyl urethane from an authentic sample of dichloroethanol yielded needles melting at 73–74° C.

These procedures were tested for the separation of the C^{14}-labeled metabolites by adding trace amounts of each radiolabeled compound (DDVP, dichloroacetaldehyde, dichloroethanol, and dichloroacetic acid) to nonradioactive samples of the other compounds and then forming the appropriate derivatives. The radioactivity in the dinitrophenylosazone accounted for all the counts from the dichloroacetaldehyde and most of the counts from DDVP but for none from the other derivatives. Similarly, the dicyclohexylamine salt contained counts only with the dichloroacetic acid-C^{14}, and the urethane only with the dichloroethanol-C^{14}.

Spectrophotometric Methods. In addition to the colorimetric method for dichloroacetaldehyde determination, the metabolism of dichloroacetaldehyde by purified alcohol dehydrogenase and by liver soluble preparations was followed by measuring the changes in DPNH concentration in the Beckman DU spectrophotometer at 340 mμ. All cuvettes contained enzyme, and readings were made against an enzyme blank.

Results

Phosphorus-Containing Metabolites from the Degradation of DDVP. DDVP-P^{32} at concentrations between 2×10^{-4} and $5 \times 10^{-3}M$ was incubated at 37° C. with whole homogenates of various tissues of rat and rabbit. The course of hydrolysis was followed by partitioning small aliquots of the incubation mixture between water and chloroform. When the hydrolysis reaction reached completion, the samples were subjected to ion exchange chromatography. With whole homogenates of liver, kidney, spleen, and adrenal glands of both the rat and rabbit, the principal DDVP metabolite was dimethyl phosphate (50 to 85%) with the remaining radioactivity appearing in desmethyl DDVP, monomethyl phosphate, and inorganic phosphate. No evidence of any other P^{32}-containing metabolites

Table IV. Effect of Cobalt on Hydrolysis of Des-Methyl DDVP by Homogenates of Rat Liver

Molarity × 10^{-5}	μL. CO_2/Min./G. Tissue
0	23
1	27
2.5	37
7.5	49
10	54
25	38
75	27
100	27

Table V. Degradation of Des-Methyl DDVP to Inorganic Phosphate and Dichloroacetaldehyde by Rat Liver Homogenate

Added Cation (2×10^{-4}M)	Inorganic Phosphate, μMoles	Dichloroacetaldehyde, μMoles
None	1.9	5.0
Co	4.3	13.2
Mg	2.5	4.4
Mn	2.6	4.2

Table VI. Effect of Substrate Concentration on Metabolism of DDVP-P^{32} by Rat Liver Homogenates

Form of Recovery	Substrate Concentration (M): 5×10^{-4}	2×10^{-2}
	Per Cent P^{32} Recovered	
Nonhydrolyzed (DDVP)	0.00	62.50
Dimethyl phosphate	68.07	35.80
Des-methyl DDVP	11.01	0.66
Monomethyl phosphate	20.92	1.02
Inorganic phosphate	0.00	0.02
Cleavage Type		
P—O—CH_3	31.9	4.5
P—O—CH=CCl_2	68.1	95.5

Table VII. Hydrolysis of Dimethyl Phosphates and Related Compounds by Rat Liver Homogenates

Compound[a]	μl. CO_2/Min./G. Tissue: Rat Plasma	Rat Liver	Rabbit Plasma	Rabbit Liver	
DDVP	7	46	27	27[b]	67[b]
Butonate	16	59	29	...	72
Chlorophan	6	35	27	29	...
DFP	8	65	39	...	98
Dibrom	13	68	11	...	27[c]
Dipterex	2[c]	19	10	6[c]	...
Phosphamidon	0.2[c]	40	0.3[c]	...	0.8[c]
Phosdrin	3	9	15	3[c]	...
Alpha-3562	0.6[c]	5[c]	3[c]	0.0	...

[a] Chemical designations for these compounds are as follows: Butonate, dimethyl 2,2,2-trichloro-1-*n*-butyryloxyethylphosphonate; Chlorophan, 2,2-dichloro-1-methoxyvinyl dimethyl phosphate; DFP, diisopropyl phosphorofluoridate; Dibrom, 1,2-dibromo-2,2-dichloroethyl dimethyl phosphate; Dipterex, dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate; Phosphamidon, 2-chloro-2-diethylcarbamoyl-1-methylvinyl dimethyl phosphate; Phosdrin, methyl 3-hydroxycrotonate dimethyl phosphate; alpha-3562, 3-hydroxy-*N*,*N*-dimethylcrotonamide dimethyl phosphate, alpha isomer.

[b] The two columns of figures for rabbit liver were derived from homogenates from different animals.

[c] The over-all hydrolysis rate for these compounds at $1 \times 10^{-2}M$ mixed with the same level of DDVP was markedly less than the rate with DDVP alone. In cases not so designated the over-all rate with the mixture was equal to or greater than the rate with DDVP alone.

Table VIII. Metabolism DDVP-C^{14} by Soluble Preparations from Rat Liver

Derivative	C^{14} Recovered as Total C.P.M. and (%)[a]: Enzyme alone	Enzyme + DPN	Enzyme + DPNH	No enzyme
2,4-Dinitrophenylosazone of glyoxal formed from dichloroacetaldehyde	14,826 (97.6)	8,400 (54.9)	5,488 (35.9)	15,288 (100)
Dicyclohexylamine salt of dichloroacetic acid	278 (1.8)	196 (1.3)	462 (3.0)	0 (0)
Phenyl urethane of dichloroethanol	...	...	11,460 (75.0)	661 (4.3)

[a] Results are not corrected for self-absorption of C^{14} by the plated derivatives.

was obtained, although the chromatographic characteristics of dichlorovinyl phosphate were not known. In the plasma of both species dimethyl phosphate accounted for 98 to 100% of the DDVP hydrolyzed. When dimethyl phosphate-P^{32} was incubated with either liver homogenates or plasma of the rat, all the radioactivity was recovered as unchanged dimethyl phosphate.

Activity of Tissues in the Hydrolysis of DDVP and Des-Methyl DDVP. The activities of rat and rabbit tissues in the hydrolysis of DDVP and des-methyl DDVP were compared by the manometric method. The enzyme preparation was placed in the side arm of the 10-ml. Warburg flasks; all other components were placed in the main compartment. Final concentrations were 4% tissue homogenate, $1 \times 10^{-2}M$ substrate, $0.0375M$ sodium bicarbonate, and $0.0164M$ sodium chloride. The results, Table I, indicate the order of activity to be liver > kidney > spleen > plasma for the degradation of DDVP by rat tissue, and for the degradation of des-methyl DDVP by both rat and rabbit tissues. The order of activity for the hydrolysis of DDVP by rabbit tissues was liver > kidney > plasma > spleen. Both substrates were rapidly hydrolyzed by the liver, kidney, and spleen homogenates, but the plasma was relatively low in activity with des-methyl DDVP, as indicated by the activity ratios (des-methyl DDVP DDVP) shown in Table I.

Similar experiments were conducted on subcellular fractions of rat liver. DDVP was hydrolyzed by the soluble and mitochondrial fractions but not by the microsomes. Des-methyl DDVP was hydrolyzed by the soluble fraction but not by the microsomes or washed mitochondria.

Properties of Rat Liver Enzymes Hydrolyzing DDVP. The effect of various metal ions on the hydrolysis of DDVP by rat liver homogenates was examined. The substrate solution was placed in the side arm of the Warburg flasks, while all other substituents were placed in the main compartment. Final concentrations were 4% liver homogenate, $1 \times 10^{-2}M$ substrate, $1 \times 10^{-3}M$ metal ion added as the chloride, $0.0375M$ sodium bicarbonate, and $0.0164M$ sodium chloride. The following preparations were compared: a freshly prepared homogenate of rat liver; a freshly prepared homogenate with $1 \times 10^{-4}M$ (ethylenedinitrilo)tetraacetic acid, disodium salt, (EDTA-Na_2) added to the homogenizing medium; and a rat liver homogenate dialyzed against $1 \times 10^{-4}M$ EDTA-Na_2 for 18 hours. The results, Table II, indicate that calcium and manganese are stimulatory, while magnesium, strontium, and cobalt are without effect or are inhibitory.

Similar experiments (Table III) with the rat liver soluble and mitochondrial fractions were expressed on the basis of the weight of tissue from which the subcellular fraction in question was derived —i.e., gram equivalent fresh weight. Only calcium ions stimulated the mitochondrial hydrolysis, while manganese ions were the principal activator for the soluble fraction.

DDVP can be hydrolyzed initially at either the P—O—vinyl or P—O—methyl grouping. The initial product from the former reaction is dimethyl phosphate, which is not metabolized further; from the latter, des-methyl DDVP, which is further degraded by rat liver prepara-

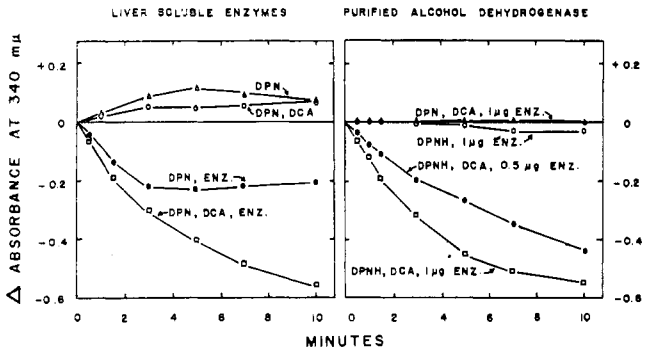

Figure 3. Change in DPNH concentration in the presence of dichloroacetaldehyde and purified alcohol dehydrogenase or rat liver soluble enzymes

DPN and DPNH are oxidized and reduced forms of diphosphopyridine nucleotide; DCA is dichloroacetaldehyde; liver soluble enzymes were 0.05 ml. of $S_{105,000}$

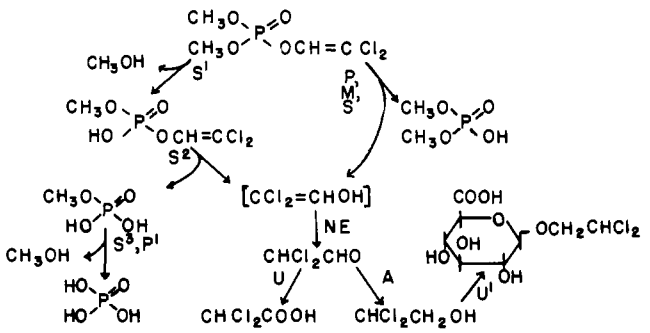

Figure 4. Metabolic pathways of DDVP in the rat based on in vitro studies

P. Plasma enzyme hydrolyzing DDVP to dimethyl phosphate, activators not studied
P[1]. Plasma enzyme hydrolyzing monomethyl phosphate to inorganic phosphate, activators not studied
S. Soluble liver enzyme hydrolyzing DDVP to dimethyl phosphate, activated by Mn^{++}
S[1]. Soluble liver enzyme hydrolyzing DDVP to des-methyl DDVP, activators not studied
S[2]. Soluble liver enzyme hydrolyzing des-methyl DDVP to monomethyl phosphate, activated by 1×10^{-4} *M* Co^{++}
S[3]. Soluble liver enzyme hydrolyzing monomethyl phosphate to inorganic phosphate, no known activators, inhibited by —SH inhibitors, pH optimum 6.8–7.2
M. Liver mitochondrial enzyme hydrolyzing DDVP to dimethyl phosphate, activated by Ca^{++}
A. Reduction of dichloroacetaldehyde to dichloroethanol by alcohol dehydrogenose, requires DPNH
NE. Nonenzymatic
U and *U*[1]. Pathway probably present, nature of enzymes not studied

tions. The nature of the initial hydrolysis of DDVP by the mitochondrial and soluble enzymes was investigated by examining the hydrolysis products of DDVP-P^{32} produced by these two enzyme preparations (typical experiment shown in Figure 2). The product of the mitochondrial enzyme(s) was almost entirely dimethyl phosphate, while with the soluble enzymes, des-methyl DDVP and monomethyl phosphate were also formed. This indicates that in the mitochondria the only significant hydrolysis is at the P—O—vinyl bond, whereas in the soluble fraction both the P—O—vinyl and P—O—methyl are attacked.

The effect of metal ions on the nature of the products formed from DDVP by the subcellular fractions was examined by ion exchange chromatography. Manganese ion added to the soluble enzymes increased the production of dimethyl phosphate and decreased the proportion of des-methyl DDVP and monomethyl phosphate. Calcium ion added to mitochondrial preparations only served to increase the rate of production of dimethyl phosphate. These divalent cations appeared to activate only the enzymes splitting the P—O—vinyl bond.

Hydrolysis of Des-Methyl DDVP by Rat Liver Enzymes. Experiments on the localization of activity and the effect of metal ions on the activity of enzymes hydrolyzing des-methyl DDVP were carried out in exactly the same manner as those for DDVP. Des-methyl DDVP hydrolysis occurred only with the soluble fraction. With this fraction cobalt ion at $1 \times 10^{-3}M$ might be stimulatory, while similar concentrations of calcium, magnesium, manganese, and strontium ions had no effect. Further investigation revealed that stimulation by cobaltic ion was marked only in the 2.5×10^{-5} to $2.5 \times 10^{-4}M$ concentration region (Table IV). Although the activity of different preparations varied in the absence of cobalt, the stimulation resulting from $1 \times 10^{-4}M$ cobalt was constant at 2.4 times in each experiment.

The initial hydrolysis of des-methyl DDVP might occur at either ester site. If the initial attack were at the P—O—methyl linkage, then both inorganic phosphate and dichloroacetaldehyde would be produced in equimolar quantities in the hydrolysis of the product, 2,2-dichlorovinyl phosphate. If P—O—vinyl cleavage were the initial step, then dichloroacetaldehyde would be produced in the first and inorganic phosphate in the second reaction. Evidence presented below indicates that monomethyl phosphate hydrolysis in these enzyme preparations is slow, and therefore, if the second mechanism is operating, the amount of dichloroacetaldehyde formed will be in excess of the amount of inorganic phosphate. This was examined by a colorimetric determination of dichloroacetaldehyde and inorganic phosphate. The incubation mixture contained the following constituents: 4% rat liver homogenate (prepared initially as a 20% homogenate in tris-HCl buffer); 20-μmoles des-methyl DDVP; $2 \times 10^{-4}M$ cobalt, calcium, or manganese chlorides; and tris-HCl buffer, pH 7.4, 0.02*M*. The final volume was 5.0 ml., and the tubes were incubated for 60 minutes at 37° C. The reaction was stopped by the addition of 2.0 ml. of 10% trichloroacetic acid, and the precipitated protein removed by centrifugation. Aliquots of the supernatant were taken for dichloroacetaldehyde determination (37° C., 1 hour, Figure 1) and inorganic phosphate analysis. All necessary controls and standard curves were run simultaneously with the experimental samples. The results, Table V, show two to three times more dichloroacetaldehyde formed than inorganic phosphate. The stimulation by $2 \times 10^{-4}M$ cobaltic ion is also apparent.

Hydrolysis of Monomethyl Phosphate by Rat Liver Enzymes. The hydrolysis of monomethyl phosphate to inorganic phosphate was investigated by procedures similar to those used in the experiments on des-methyl DDVP hydrolysis. With 20 μmoles of monomethyl phosphate as substrate, only 1.5 μmoles of inorganic phosphate were released in 2 hours of incubation. No stimulation of this slow breakdown resulted with 1×10^{-3} to $1 \times 10^{-5}M$ added calcium, cobalt, magnesium, manganese, or strontium ions. The enzyme(s) was localized in the soluble part of the cell, and the pH optimum for hydrolysis was between 6.8 and 7.2. Mercuric and silver ions, *p*-chloromercuribenzoate, and *o*-iodosobenzoate were effective inhibitors of the hydrolysis, the per cent inhibition with the inhibitors at $2 \times 10^{-4}M$ being 64, 64, 43, and 14, respectively.

An enzyme which hydrolyzed monomethyl phosphate was also present in rat plasma. On an equivalent fresh weight basis, the liver was about five times more active than the plasma.

Effect of Substrate Concentration on Nature of Products Formed in DDVP Hydrolysis by Rat Liver. Dimethyl

phosphate produced by the metabolism of the P—O—vinyl bond is not metabolized further, while des-methyl DDVP produced by hydrolysis of the P—O—methyl bond is further degraded to monomethyl and inorganic phosphates. The two types of hydrolysis to which the DDVP molecule is subjected can therefore be determined from the ratio of dimethyl phosphate formed to the total amount of des-methyl DDVP, monomethyl phosphate and inorganic phosphate formed. DDVP-P^{32} at two different substrate levels was hydrolyzed by whole homogenate of rat liver, and the metabolic products were separated by ion exchange chromatography, Table VI. The metabolic pathway initiated by P—O—methyl hydrolysis was reduced from 31.9% at a substrate concentration of $5 \times 10^{-4}M$ to 4.5% at $2 \times 10^{-2}M$ DDVP.

Hydrolysis of Dimethyl Phosphates and Related Compounds by Liver Homogenates and Plasma of Rat and Rabbit. A manometric determination of the initial hydrolysis rate for several organophosphorus esters was carried out in the same way as the experiments shown in Table I. The substrate concentration was $1 \times 10^{-2}M$ and in all cases was initially rate limiting. The results, in Table VII, show that DDVP, Butonate, Dibrom, Chlorophan, and DFP were hydrolyzed rapidly by liver and plasma of both species. Dipterex, alpha-3562, and Phosdrin were hydrolyzed more slowly or not at all. Phosphamidon was degraded rapidly by the rat liver preparation, but very slowly by rabbit liver or either plasma sample. Each substrate at $1 \times 10^{-2}M$ was then mixed with DDVP at $1 \times 10^{-2}M$ and the over-all hydrolysis rate determined. With many such substrate mixtures, the over-all rate was much less than with DDVP alone.

Metabolism of Dichloroacetaldehyde by Alcohol Dehydrogenase and the Fate of the Dichlorovinyl Portion of DDVP-C^{14}. In the presence of purified alcohol dehydrogenase or rat liver soluble preparation, dichloroacetaldehyde causes a rapid oxidation of DPNH (Figure 3). Compounds noted in Figure 3, when present, were in the following amounts: 0.05 ml. of soluble enzyme (prepared from a 20% rat liver homogenate), or 0.5 to 1.0 μg. of purified alcohol dehydrogenase; 3.77 μmoles of DPN or DPNH; and 1.47 μmoles of dichloroacetaldehyde; total reaction volume 3.0 ml. Similar experiments in which the disappearance of dichloroacetaldehyde was measured by the 2,4-dinitrophenylhydrazine method showed a parallel decrease in dichloroacetaldehyde concentration. Dichloroacetaldehyde could be metabolized almost completely, indicating that the equilibrium in this reaction, as with acetaldehyde, results in almost complete conversion to the corresponding alcohol.

DDVP-C^{14} was also incubated with soluble preparations of rat liver and the nature of the products determined, Table VIII. Reaction mixtures contained constituents, when present, in the following amounts: 1.0 ml. of soluble enzymes prepared from 20% rat liver homogenate; 7.5 μmoles of DPN or DPNH; approximately 15,000 c.p.m. DDVP-C^{14}; total reaction volume 5.0 ml. All flasks were incubated for 1 hour at 37° C. When the dinitrophenylosazone and the dicyclohexylamine salt were prepared, the reaction was stopped by addition of 2.0 ml. of $2N$ hydrochloric acid to the flask. After centrifugation, an aliquot of the supernatant was taken for dinitrophenylosazone formation and the remainder used for salt formation. The contents of the flasks to be used for phenyl urethane formation were extracted directly with benzene. The radioactivity present in the dinitrophenylosazone resulted from both dichloroacetaldehyde and unreacted DDVP. Several preliminary experiments showed that almost complete DDVP hydrolysis occurred under these reaction conditions (100° C., 30 minutes). The results of a typical experiment, Table VIII, indicate that in rat liver soluble preparations the principal fate of the dichloroacetaldehyde, released on DDVP hydrolysis in the presence of DPNH, is reduction to dichloroethanol, probably by alcohol dehydrogenase. A very small amount appears as an acid derivative, probably as dichloroacetic acid.

Discussion

The metabolic pathway of DDVP in the rat as deduced from in vitro studies and the information available on the enzymes responsible are summarized in Figure 4. Analogous, but not strictly comparable, complexes of enzymes for the primary attack on other organophosphates have been described. For the rat, there are at least three distinct enzymes in the liver and one in the plasma which hydrolyze DFP (*23*, *26*). The hydrolysis of Sarin (*O*-isopropyl methylphosphonofluoridate) and Tabun (*O*-ethyl *N*,*N*-dimethylphosphoramidocyanidate) seems also to be carried out by several different enzymes in plasma and tissues of mammals (*5*, *10*, *26*). Metal ion activation studies on the enzymes hydrolyzing DFP, Sarin, and Tabun (*6*, *10*, *23*, *24*) have yielded complex results and do not seem applicable at present to DDVP hydrolysis. It may be noted that manganese is the most common activator and that the particulate enzyme hydrolyzing DFP as in the case of DDVP requires calcium (*23*).

Previous work on enzymes hydrolyzing organophosphorus antiesterase agents has considered primarily or entirely the hydrolysis of the anhydride bond. Such hydrolysis with DDVP would yield dimethyl phosphate, a product which was not metabolized further with rat liver homogenates or plasma and was excreted unchanged when administered to rats (*8*, *29*). The alternate cleavage of the P—O—alkyl ester bond is known to occur in vivo with DDVP (*8*) and many other organophosphates (*12*, *19*, *28*, *29*). In the present investigation different enzymes were found to effect hydrolysis of the P—O—vinyl anhydride and the P—O—methyl ester bonds. The subcellular localizations and metal ion activators served to differentiate the enzymes. Preliminary results on ammonium sulfate fractionation of the soluble liver fraction showed that the enzymes hydrolyzing the P—O—methyl precipitated predominately between 60 and 80% saturation, while the P—O—vinyl hydrolyzing esterases precipitated between 40 and 60% saturation. The change in the ratio of hydrolysis at the two sites in the DDVP molecule when different substrate levels are present may indicate that the enzyme effecting the cleavage of the P—O—methyl linkage is saturated at a lower substrate level or is subject to inhibition by excess substrate or by products of either cleavage.

At least three enzymes in rat liver are involved in DDVP hydrolysis and three in DFP hydrolysis. Where such a complex enzyme mixture is acting, no definitive results can be obtained with mixed substrate experiments, unless the enzymes are extensively purified so that they can be individually assayed. In the cases considered in Table VII where the DDVP hydrolysis was markedly diminished in the presence of another organophosphate ester, it is probable that this second ester associated with at least some of the same enzymes hydrolyzing DDVP. No evidence was obtained in the present study to suggest that DDVP and related phosphates are split by more than one enzyme in plasma and that this enzyme is not the A-esterase which is known to split DFP and diethyl *p*-nitrophenyl phosphate (*1*, *22*).

The metabolism of a vinyl phosphate, Chlorophan, differing from DDVP by an alpha methoxyl rather than an alpha hydrogen, has been investigated by manometric techniques (*14*). Plasma and liver were particularly active in its hydrolysis, but metabotites were not characterized.

The fate of the nonphosphorus-containing residues released on metabolism of DDVP was partially clarified. Methanol metabolism has been extensively investigated (*33*). The metabolism of dichloroacetaldehyde, produced on hydrolysis of DDVP or des-methyl DDVP, by purified alcohol dehydrogenase or soluble preparations from rat liver is very similar to that of chloral (*11*). The

formation and excretion of dichloroethyl glucuronide is known to occur in rabbits administered dichloroethanol (*31*) and in rats administered DDVP (*8*). The observation that labeled carbon dioxide is produced in vivo in rats from DDVP-C^{14} (*8*) indicates that the further metabolic reactions shown in Figure 4 do not account for the complete metabolism of DDVP. The mechanism by which the small amount of dichloroacetic acid observed is produced has not been investigated. An enzyme, which requires DPN, has been described for the metabolism of chloral to trichloroacetic acid (*13*).

The method described for the colorimetric determination of dichloroacetaldehyde might be adapted for residue analysis of certain organophosphorus insecticides. DDVP, Dipterex, and Butonate form blue derivatives which appear to be identical with that formed from dichloroacetaldehyde, whereas most natural aldehydes and ketones yield red derivatives. Under these reaction conditions with 2,4-dinitrophenylhydrazine, Dibrom gave a colored derivative with a different absorption maximum, one very similar to that given by chloral. Presumably acid degradation of these compounds results in carbonyl compounds which react to form dinitrophenylosazones. By suitable chromatography and/or solvent partitioning this method might be made specific for DDVP. The sensitivity might approach the 1-μg. range by modification of the reaction times and volumes, or by increasing the extent of reaction by carrying out the hydrolysis first in dilute alkali and then reacting the product with 2,4-dinitrophenylhydrazine, or by conducting the acid hydrolysis in sealed tubes.

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INSECTICIDE ANALYSIS

Infrared Analysis of Insecticides to Determine Toxaphene Alone or in the Presence of Dichlorodiphenyltrichloroethane (DDT)

WILLIAM H. CLARK

Research Center, Hercules Powder Co., Wilmington, Del.

TOXAPHENE is used mainly in emulsifiable concentrates and wettable powders and dusts. To prepare these materials for analysis by infrared absorption, toxaphene and other organic insecticides are separated from inert ingredients by a simple chromatographic procedure for emulsifiable concentrates and by methanol extraction of dusts. These separations gave recoveries of 98% of the active ingredients in several commercial formulations.

Infrared absorption spectroscopy is useful in the analysis of insecticides and pesticides because it offers a simple, accurate, and specific assay of multicomponent mixtures (*1, 2*).

Toxaphene, which is chlorinated camphene containing 67 to 69% chlorine, can be determined quantitatively in this way. A quantitative infrared method for the determination of toxaphene is described herein. A previous article reported the use of infrared spectroscopy for the identification of toxaphene (*5*).

There are three general methods for determination of toxaphene: measurement of total chloride (*6*), colorimetric methods (*4*), and infrared absorption.

The total chloride method has been widely used for both assay and residue work, but it lacks specificity. The major advantage of the colorimetric method is its sensitivity, which makes it useful in residue determinations. The infrared absorption method offers the advantage that it is always specific for toxaphene in the presence of DDT