Å [between molecules at (x,y,z) and $(x,y,z-1)$]. None of these are appreciably smaller than the sum of corresponding van der Waals radii (Pauling, 1960), and hence do not represent significant interactions which would modify the geometry about the phosphorus; the distortions found should represent a molecular property and not the consequence of packing forces within the crystal structure.

That is, owing to the lack of intermolecular interactions, the molecular geometry should be very close to the absolute ground-state geometry. The rotational degree of freedom about the C-8–O-6 bond is minimized. Consequently, the phosphorus position, and therefore the intramolecular distances involving phosphorus, are within a small range of possible values. Barring unusual solvent interactions, the solid state geometry should also be very close to that in vivo.

Supplementary Material Available: A listing of the observed and calculated structure factor amplitudes (six pages). Ordering information is given on any current masthead page.

LITERATURE CITED

Bally, R., *Acta Crystallogr., Sect. B* **26**, 477 (1970).
Baughman, R. G., Jacobson, R. A., *J. Agric. Food Chem.* **23**, 811 (1975).
Bergmann, F., Segal, R., *Biochem. J.* **58**, 692 (1954).
Bergmann, F., *Discuss. Faraday Soc.* **20**, 126 (1955).
Busing, W. R., Martin, K. O., Levy, H. A., "OR FLS, A Fortran Crystallographic Least Squares Program", U.S. Atomic Energy Commission Report ORNL-TM-305, Oak Ridge National Laboratory, Oak Ridge, Tenn., 1962.
Busing, W. R., Martin, K. O., Levy, H. A., "OR FEE, A Fortran Crystallographic Function and Error Program", U.S. Atomic Energy Commission Report ORNL-TM-306, Oak Ridge National Laboratory, Oak Ridge, Tenn., 1964.
Canepa, F. G., Pauling, P., Sörum, H., *Nature (London)* **210**, 907 (1966).
Clark, V. M., Hutchinson, D. W., Kirby, A. I., Warren, S. G., *Angew. Chem.* **76**, 704 (1964).
Collin, R. L., *J. Am. Chem. Soc.* **88**, 3281 (1966).
Dickens, B., Prince E., Schroeder, L. W., Jordon, T. H., *Acta Crystallogr., Sect. B* **30**, 1470 (1974).
Fukuto, T. R., *Bull. W. H. O.* **44**, 31 (1971).
Fukuto, T. R., Metcalf, R. L., *J. Agr. Food Chem.* **4**, 930 (1956).
Furberg, S., *Acta Chem. Scand.* **9**, 1557 (1955).
Hansch, C., Deutsch, E. W., *Biochim. Biophys. Acta* **126**, 117 (1966).
Hanson, H. P., Herman, F., Lea, J. D., Skillman, S., *Acta Crystallogr.* **17**, 1040 (1960).
Hohme, E., Lohs, K., *Z. Naturforsch. B* **24**, 1071 (1969).
Hubbard, C. A., Quicksall, C. O., Jacobson, R. A., "The Fast Fourier Algorithm and the Programs ALFF, ALFFDP, ALFFPROJ, ALFFT, and FRIEDEL", U.S. Atomic Energy Commission Report IS-2625, Iowa State University and Institute for Atomic Research, Ames, Iowa, 1971.
Jacobson, R. A., "An Algorithm for Automatic Indexing and Bravais Lattice Selection. The Programs BLIND and ALICE", U.S. Atomic Energy Commission Report IS-3469, Iowa State University and Institute for Atomic Research, Ames, Iowa, 1974.
Johnson, C. A., "OR TEP-II: A Fortran Thermal-Ellipsoid Plot Program for Crystal Structure Illustrations", U.S. Atomic Energy Commission Report ORNL-3794 (Second Revision with Supplemental Instructions), Oak Ridge National Laboratory, Oak Ridge, Tenn., 1971.
Klyne, W., Prelog, V., *Experientia* **16**, 521 (1960).
Krupka, R. M., *Can. J. Biochem.* **42**, 677 (1964).
Lai, T. F., Marsh, R. E., *Acta Crystallogr., Sect. B* **30**, 1570 (1974).
Lawton, S. L., Jacobson, R. A., *Inorg. Chem.* **7**, 2124 (1968).
Main, P. M., Woolfson, M. M., Germain, G., "MULTAN: A Computer Program for the Automatic Determination of Crystal Structures", Department of Physics, University of York, York, England, 1971.
O'Brien, R. D., "Toxic Phosphorus Esters", Academic Press, New York, N.Y., 1960, p 257 ff.
O'Brien, R. D., *J. Agric. Food Chem.* 11, 163 (1963).
Pauling, L., "Nature of the Chemical Bond", Cornell University Press, Ithaca, N.Y., 1960.
Templeton, D. H., in "International Tables for X-ray Crystallography", Vol. III, The Knoch Press, Birmingham, England, 1962, pp 215–216, Table 3.3.2c.
Williams, D. E., "LCR-2, A Fortran Lattice Constant Refinement Program", U.S. Atomic Energy Commission Report IS-1052, Iowa State University and Institute for Atomic Research, Ames, Iowa, 1964.

Received for review July 14, 1975. Accepted October 29, 1975. Prepared for the U.S. Energy Research and Development Administration under Contract No. W-7405-eng-82.

Analysis of Phosphorus-Containing Hydrolytic Products of Organophosphorus Insecticides in Water

Christian G. Daughton, Donald G. Crosby, Richard L. Garnas, and Dennis P. H. Hsieh*

A new approach to the analysis of ionic dialkyl phosphates and thiophosphates in large volumes of aqueous media by using Amberlite XAD-4 resin is presented. Recoveries for diethylphosphoric acid and diethylthiophosphoric acid at 0.01–0.1 ppm in 500 to 4000 ml of aqueous media were 100 and 85%, respectively; recoveries for dimethylphosphoric acid and dimethylthiophosphoric acid at 0.1 ppm in 500 ml of aqueous media were 50 and 97%, respectively. Several easy methods are described for the elimination of possible interference due to inorganic phosphate and the potential application for urine analysis is discussed. All four ionic dialkyl phosphates were shown to be nearly as inhibitory as parathion to human plasma cholinesterase.

The fate of organophosphorus insecticides in the environment has been the subject of numerous investigations and is currently an important issue in worker-reentry studies in California (Spear et al., 1975). Chemical and enzymatic hydrolysis of these insecticides generally results in formation of a dialkylphosphoric acid or dialkylphosphorothioic acid. Efficient methods for the isolation and analysis of these highly polar and acidic hydrolytic products in field samples are at present not available.

Diesters of phosphoric acid occur naturally in biological systems (dinucleotides and phosphoglycerides). Phosphorus and sulfur from organophosphorus insecticides are

Department of Environmental Toxicology, University of California, Davis, California 95616.

assimilated by microorganisms (Gunner, 1970), and the phosphorus from dialkyl phosphates is incorporated into biological molecules as inorganic phosphate (Dorough and Arthur, 1961; Wolfenden and Spence, 1967). Otherwise, little is known about the environmental fate of the synthetic alkyl phosphates (Yu and Sanborn, 1975).

Hydrolysis of the organophosphorus insecticides is considered as a detoxication, the dialkyl phosphate moieties being assumed nontoxic (O'Brien, 1967; Eto, 1974; Zech and Wigand, 1975) even though their potential toxicities have received little attention.

Ionic dialkyl phosphates are used commercially in a variety of products as well as mineral flotation agents in ore processing (Van Wazer, 1961). Phosphorus pesticides are extensively used, over 5 million pounds having been applied in California in 1973 (Pesticide Use Report, 1974); in addition, the *O,O*-dialkylphosphorus hydrolysis products are stable toward both acid and base (Eto, 1974). These ionic dialkyl phosphates would not undergo rapid photochemical alterations since they only weakly absorb the uv portion of sunlight (Halmann and Platzner, 1965) and, as they are highly water-soluble and nonvolatile salts, there is ample justification for concern about their environmental fate.

Presented here is a procedure for isolating and determining, in large volumes of aqueous media, ionic diethyl phosphate, diethyl thiophosphate, dimethyl phosphate, and dimethyl thiophosphate suitable for application in environmental monitoring and perhaps urine analysis. Procedures for eliminating interference due to inorganic phosphate are also discussed.

EXPERIMENTAL SECTION

Apparatus and Glassware. Amberlite XAD-4 (Rohm and Haas, Philadelphia, Pa.) was cleaned by sequential overnight Soxhlet extractions with acetone and methanol. The clean resin (100 ml), in a 250-ml separatory funnel fitted with a glass wool plug before the stopcock, was conditioned by rinsing with several volumes of distilled water. A 500-ml separatory funnel served as the sample reservoir and was connected through a Neoprene stopper to the resin bed. The stopper did not contact the sample, since the aqueous level was maintained about 2 cm above the resin.

Evaporations in vacuo were performed on a Cal Lab rotary evaporator (Berkeley, Calif.); pH was measured with a Sargent-Welch Model NX pH meter equipped with a combination electrode; extractions were performed in graduated 50-ml conical screw-cap Pyrex centrifuge tubes by vortexing on a Scientific Products Deluxe Vortex Mixer for 1 min.

Gas chromatographic (GC) analyses employed a Packard Model 417 gas chromatograph equipped with a phosphorus thermionic detector and a glass column, 1.8 m × 2 mm i.d., packed with equal parts 15% QF-1 and 10% DC-200 on Gas-Chrom Q (80–100 mesh) at 140 °C; injector, 200 °C; detector, 250 °C; nitrogen, 22 ml/min; air, 230 ml/min; hydrogen, 55 ml/min. Mass spectral confirmation of the methyl esters, using the data of Safe and Hutzinger (1973) and Eto (1974), employed a Varian Aerograph Series 1400 GC equipped with a glass column of 3% OV-1 on Chromosorb W interfaced with a Finnigan Model 3000 Peak Identifier (GC–MS).

Reagents. Ammonium molybdate tetrahydrate (MCB, reagent grade) aqueous solution contained 0.30 g/ml and was stored in a polyethylene bottle. Safranine reagent contained 0.15 g/ml of water of Safranine-O biological stain (3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride, MCB). Instant Ocean Synthetic Sea Salt was obtained from Aquarium Systems, Inc. (Eastlake, Ohio). Ethereal ethanolic diazomethane was prepared from Diazald reagent (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co.) using an Aldrich Diazald Kit Diazomethane Generator, according to instructions, except that the distilled diazomethane was collected in a dry ice bath, and stored at −20 °C in a dark glass bottle with a poly-seal cap. (**Caution**: Diazomethane is highly explosive and toxic.)

Enzyme–buffer solution used in the cholinesterase inhibition determination (25 ml of enzyme/150 ml of buffer) contained human plasma cholinesterase (Sigma Chemical Co., reference serum for SMA procedures, Product No. R3626) and was reconstituted according to instructions. Buffer contained sodium barbital (7.42 g), KCl (84.46 g), and KH_2PO_4 (1.089 g) diluted to 1000 ml with distilled water and the final pH adjusted to 8.0 with HCl. Acetylcholine reagent was 5% acetylcholine bromide (MCB, mp 143–145 °C) in distilled water. Incubations were done in 25-ml test tubes in duplicate, each containing a 7-cm glass agitation rod. Tubes were incubated at 30 °C in a New Brunswick Scientific Gyrotory Shaker Bath no. G76 fitted with a test tube rack and agitated at 120 rpm.

Stock solutions of standards were prepared from the following compounds (equivalent to 100 mg of the free acid), weighed into separate 100-ml volumetric flasks and diluted to volume with acetone: diethylphosphoric acid (DEP) (Eastman Kodak Co., Rochester, N.Y.), potassium diethyl thiophosphate (DETP) (American Cyanamide Co., Princeton, N.J.), dimethylphosphoric acid (DMP) (American Cyanamide Co.), potassium dimethyl thiophosphate (DMTP) (American Cyanamide Co.), and potassium phosphate dibasic (Analytical Reagent). (The potassium salts of the thioates are soluble in acetone and also need not be acidified for methylation.) All stock solutions were stored at −20 °C.

For GC, an appropriate amount of each standard was measured with a microsyringe into a 25-ml volumetric flask, and 10 drops of methanol were added followed by diazomethane until the yellow color persisted; after 2 min, acetic acid reagent (1% glacial acetic acid in hexane) was added dropwise to eliminate excess diazomethane, followed by dilution with hexane.

General Procedure for Determination of Ionic Dialkyl Phosphates in Aqueous Media. The resin bed was acidified with several volumes of distilled water adjusted to pH 1.25 with concentrated HCl, and the aqueous sample (pH 1.25) allowed to flow through at about one l./h. At this pH, DETP and DEP were stable for at least 3 days, so a slower flow rate or delay in the subsequent procedure would be acceptable. The effluent containing inorganic salts was discarded and the resin bed aspirated by vacuum to remove as much residual solution as possible.

Analytes were eluted from the resin with four 75-ml portions of acetone and the combined eluates evaporated in vacuo at 25 °C until about 30 ml of aqueous residual remained. The resin served to preconcentrate the compounds of interest from large initial volumes (for example, 4 l.) to about 30 ml. The concentrate was evaporated in vacuo, at 55 °C, to dryness or near dryness after adjusting the pH to 7.0 with 0.1 and 1.0 N NaOH. The nonvolatile salts will not codistill with water and are stable at 55 °C. With a minimum amount of distilled water (no more than two 2-ml portions), the salts were redissolved and transferred to a 50-ml centrifuge tube, the flask rinsed with 5 ml of ethyl acetate which also was transferred to the centrifuge tube, and NaCl was added to saturation together with enough HCl to lower the pH to 0.5. The mixture was

vortexed for 1 min, and the organic layer transferred to a 25-ml volumetric flask, and the rinsing and extraction were repeated with another 5 ml of ethyl acetate; at least two more extractions are necessary if DMP is present. The combined extracts were methylated as delineated under Reagents and an aliquot of the methylated extract was injected on the GC; quantitation was by interpolation from the linear regression equation yielded by the peak heights of standards (i.e., the *O*-methyl esters of the phosphates and *S*-methyl esters of the thiophosphates).

Elimination of Interference Due to Inorganic Phosphate. The esterification of inorganic phosphate (P_i) to yield trimethyl phosphate (TMP) interferes with quantitation of DMP, the methyl ester of which is TMP. This has been a major problem, especially for the analysis of urine which contains large amounts of phosphate-bearing compounds where acidification, prior to solvent extraction, liberates up to 800 mg/l. of P_i. Although P_i does not partition favorably into organic solvent, even the small percentage which partitions represents a large interference. Several procedures to eliminate P_i prior to solvent extraction follow.

Procedure 1 (For Routine Use). After the sample has passed through the resin, and the bed has been fully aspirated, the aqueous residual retained by the resin still contains P_i in its original concentration since P_i is not adsorbed. When present at less than 530 ppm, P_i can be totally removed from the resin by rinsing with three 50-ml portions of distilled water at pH 1.25. The amount of residual solution can also be decreased by using less resin. Following aspiration of the rinse water, the procedure can be continued as outlined above.

Procedure 2 (For Further Cleanup). After the acetone has been evaporated from the resin eluate, and is no longer detectable by odor, sufficient ammonium molybdate is added to form the yellow heteropolyacid, phosphomolybdate, according to the reaction: $PO_4^{3-} + 12MoO_4^{2-} + 3NH_4^+ + 24H^+ \rightarrow (NH_4)_3PO_4 \cdot 12MoO_3 + 12H_2O$.

This complex can be precipitated by high molecular weight cations such as safranine (Suzuki et al., 1968) and then filtered from solution using a fine-porosity fritted glass filter, from which the precipitate is easily cleaned with acetone and NH_4OH. Safranine is added to the sample at pH <2.0 until the filtrate is slightly pink, indicating the total precipitation of phosphomolybdate. Excess safranine offers no interference in the further procedure, although excess molybdic acid will consume diazomethane.

Procedure 3. Alternatively, after formation of the phosphomolybdate, the ionic dialkyl phosphates can then be partitioned from the aqueous sample with several twofold volumes of $CHCl_3$. Phosphomolybdate is not soluble in chlorinated hydrocarbon solvents. The bottom phase can then be methylated. (Note—The chlorinated solvents are not compatible with the thermionic detector employed in this procedure.)

Potential Application of the General Procedure for Ionic Dialkyl Phosphates in Urine. The use of large samples (i.e., several hundred milliliters if desired) allows for detection of much lower concentrations of compound in the original sample. Since the resin irreversibly adsorbs urinary pigments, the urine was precleaned by the following procedure. A suitable column was packed with enough 80/100 mesh Florisil to effectively decolorize the sample (e.g., 20 ml for 200 ml of urine), and was prewashed with several volumes of pH 1.8 water to allow optimum decolorization. The urine was centrifuged to remove solids, acidified with HCl to pH 1.25, and poured through the Florisil column. After rinsing the column with acidified water, none of the analytes were adsorbed to the Florisil and the sample could then be applied to the resin after acidifying to pH 0.5 (to liberate P_i which would otherwise be liberated during subsequent solvent extraction of the acidified concentrated sample) and then readjusting to pH 1.25. The Florisil cleanup also eliminated much of the material which causes viscosity of the urine, and excessive foaming and bumping during subsequent evaporation. After aspiration of the resin, procedures 1 and 3 were followed.

Cholinesterase Inhibition Study. Duplicate incubation tubes received 1, 10, and 100 μl of each of the following standards in redistilled acetone: 1 μg/μl of *p*-nitrophenol (MCB, mp 113–114 °C), DMP, K-DMTP, DEP, K-DETP, and parathion (Stauffer Chemical Co., 99.2%) and 1 ng/μl of paraoxon (Sigma Chemical Co., 99.9%). The blank control received 1, 10, and 100 μl of redistilled acetone. In all tubes, the acetone was partially evaporated and 3 ml of the enzyme–buffer solution added to each. After incubation for 45 min, the pH was measured, followed by the timed addition of 1 ml of the acetylcholine reagent to each tube at 1-min intervals. All of the samples were reincubated until the controls showed a pH of 6.1–6.2. At this moment, the pH of all the samples was successively taken again at 1-min intervals. The percent inhibition was calculated as [(ΔpH control − ΔpH sample)/ΔpH control] × 100.

RESULTS AND DISCUSSION

With one exception (Plapp and Casida, 1958), all available methods for analysis of ionic dialkyl phosphates involve their solvent partitioning from small, highly acidified volumes, usually less than 3 ml (St. John and Lisk, 1968; Askew et al., 1969; Shafik and Enos, 1969; Shafik et al., 1970, 1971a,b, 1973; Thompson, 1974), or from solid crop samples (Archer, 1974). From our experience, the partitioning of DEP and especially DMP from small volumes of water is not a favorable process; using the extraction procedure of Shafik and Enos (1969), Davis et al. (1973) recovered 75% of the DEP and 91% of the DETP from aqueous microsomal preparations after three successive extractions of a 2.5-ml sample. With large volumes of aqueous sample, efficient partitioning would require the use of very large quantities of expensive reagent grade solvents. Therefore, we employ a method to preconcentrate large aqueous samples to allow for partitioning from the resulting smaller sample and to eliminate the majority of P_i. Garnas et al. (1974) previously reported on the efficiency of Amberlite XAD-4 resin in the preconcentration of various aromatic pesticides and their metabolites from large volumes of water. Continuing this work, we have examined the adsorption efficiencies of the ionic dialkyl phosphates from large volumes of aqueous media and the recovery of these compounds from the resin (Daughton et al., 1975). In addition, previous procedures have experienced difficulties with inorganic phosphate occurring in the initial sample, since it interferes with DMP during GC if the methyl esters are formed, or it lengthens both sample preparation time by necessitating lengthy cleanup procedures and intersample injection times for GC when the amyl esters are formed (Shafik et al., 1973).

DMTP and DETP, due to their thiono–thiolo isomerization, yield both the *O*-methyl and *S*-methyl isomers during methylation, but a protic solvent such as methanol promotes formation of the *S*-methyl ester by over 90%. This is a major advantage in methylation of the thioates, since the higher diazoalkanes yield a mixture of *O*- and *S*-alkyl esters regardless of the presence of a protic solvent, making quantitation more difficult.

Table I. Recovery of Ionic Dialkyl Phosphates and Inorganic Phosphate from Aqueous Media[a]

Compd	Aq sample vol, l.	Concn of compd, ppm	Sample pH	% recovery
DEP	0.5	0.1	1.25-2.0	100
	0.5	0.1	8.5	50
	4.0	0.01	1.25	100
DETP	0.5	0.1	1.25-8.5	85
	4.0	0.01	1.25	85
DMTP	0.5	0.1	1.25-8.5	97
DMP	0.5	0.1	1.25	40
	0.5	0.1	1.25	50[b]
P_i	0.5	200	1.25	3[c]

[a] Synthetic sea salts and distilled water. [b] Sample saturated with NaCl prior to passage through the resin. [c] Corresponds to amount left in aqueous residual retained by 100 ml of resin.

Table II. Affinities[a] of Dialkyl Phosphates and P_i for XAD-4

Rel S content and aliphatic character[b]	pK[c]	Rel affinity[b] for XAD-4
DETP	1.49	++++
DMTP	Not available	+++
DEP	1.37	++
DMP	1.25	+
P_i	1.97	0

[a] As defined by increased recovery from the aqueous sample and decreased ease of elution from the resin with water or organic solvent. [b] In order of decreasing amount. [c] pK for first dissociation step in 7% alcohol from Fest and Schmidt (1973).

Table I lists the recoveries from 500 ml to 4 l. of distilled water and Instant Ocean synthetic seawater, fortified with 0.01-0.1 ppm of each of DEP and DETP or 0.1 ppm each of DMP and DMTP. The sample pH, prior to passing through the resin, did not affect the adsorption of DETP and DMTP, whereas DEP was efficiently adsorbed between pH 1.25 and 2.0 and only half as well as pH 8.0. Recovery of DMP was 40% at pH 1.25. It seems that DEP and DMP, having lower pK values and being more polar than the thioates, require protonation to be adsorbed. Hence, the pH may need to be further lowered or the DMP salted out with NaCl as noticed in the isolation of *p*-nitrophenol conjugates (Garnas et al., 1974); addition of NaCl increased the recovery of DMP to 50%. P_i is not adsorbed at 200 ppm and pH 1.25.

Since DMP is the most acidic of any dialkyl phosphate, it represents the extreme insofar as one considers difficulties in extraction or adsorption processes. From our experience with these four dialkyl phosphates, adsorption onto the resin is favored not only by higher pK but also by increased aliphatic character and sulfur content (Table II). For example, after adsorption of 50 μg each of DETP and DEP to the resin and rinsing with 30 l. of pH 1.25 synthetic seawater over a 6-day period, recoveries for DETP and DEP were 62 and 24%, respectively. Extrapolating the trend of pK values in Table II, one would expect the monoesters to adsorb to the resin since their pK values are higher (e.g., pK = 1.54 for monomethylphosphoric acid; Fest and Schmidt, 1973). In addition, since the pK values for diethyldithiophosphoric and dipropylphosphoric acids are 1.55 and >1.52, respectively (Fest and Schmidt, 1973), they should show even greater affinity than DETP due to their greater aliphatic character and sulfur content.

Both the amount of aqueous residual retained by the resin and the volume of elution solvent can be decreased by minimizing the volume of resin, while still allowing for adsorption of analytes. We have purposely used a rather large amount of resin, since this is where some previous attempts at isolating polar compounds from aqueous media using XAD have failed (e.g., Thompson and Markey, 1975).

Table III. Effect of Cleanup on the Recovery of Ionic DEP, DETP, and P_i from Aqueous Media

Cleanup procedure	Compds	Aq sample	Vol, ml	Concn of compd, ppm	% recovy
1	DEP	Spent microbial broth	600	0.1	>90
	DETP			0.1	>90
	P_i			530	0
2	DEP	Deionized water	500	0.1	>90
	DETP			0.1	>90
	P_i			20	0
3 + 1	DEP	Human urine	100	0.5	60
	DETP			0.5	90
	P_i			~800	0

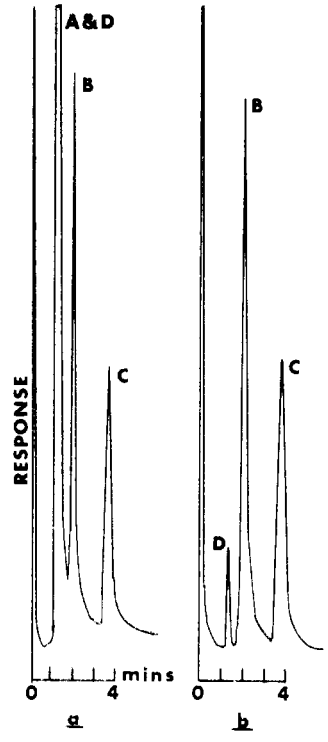

Figure 1. Gas chromatograms (500-ml sample, 0.1 ppm each of DEP and DETP, and 20 ppm of K_2HPO_4): (a) prior to cleanup with molybdic acid and safranine; (b) after cleanup; (A) trimethyl ester of inorganic phosphate; (B) *O*-methyl ester of DEP; (C) *S*-methyl ester of DETP; (D) *O*-methyl ester of DETP.

The effectiveness of cleanup procedure 1 is shown in Table III. DEP and DETP were fortified at 0.1 ppm in filtered spent microbial broth which contained 530 ppm of P_i; after aspiration of the resin, rinsing with three 50-ml portions of pH 1.2 water completely eliminated all P_i and allowed recoveries greater than 90% for DEP and DETP. Procedure 2 is effective in eliminating minor residual amounts of P_i as depicted with the two gas chromatograms in Figure 1, representing the same sample before and after elimination of P_i; as shown in Table III, this allowed for recovery of greater than 90% of DEP and DETP while totally eliminating P_i at 20 ppm. In procedure 2, quinoline can be substituted for safranine. Procedure 3 together with

Table IV. Acetylcholinesterase Inhibition

Compound	I_{50}[a]
Paraoxon	0.0008
Diethyl thiophosphate	0.7
Parathion	1.0
Dimethyl phosphate	4.8
Diethyl phosphate	9.4
Dimethyl thiophosphate	32.3
p-Nitrophenol	No inhibition

[a] Moles normalized to parathion which elicit a 50% inhibition of human plasma cholinesterase. Values represent averages of duplicate samples differing by less than 1%.

procedure 1 could be useful for urine analysis; recoveries for DEP and DETP were 60 and 90%, respectively, while residual P_i was eliminated.

The thioates are partitioned nearly quantitatively from the final aqueous sample with the first ethyl acetate extraction, but DMP and DEP behave quite differently. This step remains the limiting factor in the success of this procedure since the polar nature and acidity of DMP prevent its efficient partitioning from water. From our experience, DEP quantitatively partitions after two extractions with ethyl acetate, whereas after three extractions only 60% of DMP is recovered; contrary to published results (Shafik et al., 1973), acetonitrile–diethyl ether (1:1) gives about the same results.

Attempting to avoid solvent extraction of the final aqueous sample, the salts from the evaporated samples were transferred from the round-bottomed flasks with both acidified and nonacidified acetone or methanol giving recoveries comparable to solvent extraction for the thioates. However, the salts of DMP and DEP are not as soluble in organic solvent and must be in protonated form before they will methylate. Investigation of an alternative method of extraction, analogous to the procedure of Eisenbraun (1970), showed that the partitioning of DEP and DMP can be improved further by adding the diazomethane directly to the extraction mixture of sample and ethyl acetate and mixing with a disposable pipet. In this case, the pH is adjusted to 0.9 to prevent excessive consumption of the diazomethane. This method of combined extraction–methylation requires further investigation.

A method for analysis of the ionic dialkyl phosphates is desirable for many reasons, especially considering the lack of toxicological data on these compounds. The ionic dialkyl phosphates are generally assumed to be nontoxic, although this assumption is supported only by the surprisingly few published reports which have demonstrated the lack of various possible toxic effects concerning only ionic dialkyl dithioates (Dorough and Arthur, 1961; Augustinsson and Heimburger, 1955; Mazur, 1946; Zuckerman et al., 1970), and in one case, dialkyl thioates (Walker and Stojanovic, 1973). On the other hand, diethyldithiophosphoric acid was found to be toxic to fish (Murphy, 1972), Augustinsson and Heimburger (1955) showed the esterase-inhibiting ability of DEP, and Shafik et al. (1971b) reported the in vivo inhibition of plasma cholinesterase in rats fed DETP.

We have compared the inhibition of human plasma cholinesterase by parathion, paraoxon, and their hydrolysis products, DEP, DETP, DMP, DMTP, and *p*-nitrophenol, using a modification of the Giang and Hall ΔpH method (1951), noting the importance of temperature control (Ellin and Vicario, 1975). As shown in Table IV and as reported in the literature (Giang and Hall, 1951), paraoxon proved to be about 1000 times more inhibitory than parathion. Surprisingly, DETP and DMP were about as inhibitory as parathion; DEP and DMTP were about an order of magnitude less inhibitory than parathion, whereas *p*-nitrophenol, a compound of high toxicity itself (Gleason et al., 1969), showed no inhibition relative to the blank. The only conflict with these results is the recent report that DMTP is not inhibitory to bovine true cholinesterase (Walker and Stojanovic, 1973).

In summary, our method provides a means of analyzing aqueous samples of large volumes for the detection of the potentially toxic phosphorus-containing hydrolytic products of the widely and heavily used organophosphorus pesticides. In addition to the application of this procedure to environmental monitoring of ionic dialkyl phosphates, the use of XAD-4 resin and the P_i cleanup procedures could be applied to analyzing large volumes of urine for alkyl phosphates.

ACKNOWLEDGMENT

We thank the Residue Laboratory of this department for supplying some analytical standards.

LITERATURE CITED

Archer, T. E. *J. Agric. Food Chem.* **22**, 974 (1974).
Askew, J., Ruzicka, J. H., Wheals, B. B., *J. Chromatogr.* **41**, 180 (1969).
Augustinsson, K., Heimburger, G., *Acta Chem. Scand.* **9**, 310 (1955).
Daughton, C. G., Crosby, D. G., Garnas, R. L., Hsieh, D. P. H., 30th Annual Northwest Regional Meeting of the American Chemical Society, Honolulu, Hawaii, June 1975, AFCD 002.
Davis, J. E., Cranmer, M. F., Peoples, A. J., *Anal. Biochem.* **53**, 522 (1973).
Dorough, H. W., Arthur, B. W., *J. Econ. Entomol.* **54**, 1140 (1961).
Eisenbraun, E. J., *J. Chem. Educ.* **47**, 710 (1970).
Ellin, R. I., Vicario, P. P., *Arch. Environ. Health* **30**, 263 (1975).
Eto, M., Ed., "Organophosphorus Pesticides: Organic and Biological Chemistry", CRC Press, Inc., Cleveland, Ohio, 1974, pp 129, 193.
Fest, C., Schmidt, K. J., "The Chemistry of Organophosphorus Pesticides", Springer-Verlag, New York, N.Y., 1973, pp 62–63.
Garnas, R. L., Ross, R. D., Crosby, D. G., 88th Annual Meeting of the Association of Official Analytical Chemists, Washington, D.C., Oct 1974, paper no. 045.
Giang, P. A., Hall, S. A., *Anal. Chem.* **23**, 1830 (1951).
Gleason, M., Gosselin, R., Hodge, H., Smith, R., Ed., "Clinical Toxicology of Commercial Products", Williams and Wilkins, 1969, p 102.
Gunner, H. B., *Ghent-Rijksfaculteit Landbouwwetenschappen Mededelingen* **35**(2), 581 (1970).
Halmann, M., Platzner, I., *J. Chem. Soc.*, 5380 (1965).
Mazur, A., *J. Biol. Chem.* **164**, 271 (1946).
Murphy, S. D., in "Degradation of Synthetic Molecules in the Biosphere", National Academy of Sciences, Washington, D.C., 1972, p 322.
O'Brien, R. D., "Insecticides: Action and Metabolism", Academic Press, New York, N.Y., 1967, p 46.
"Pesticide Use Report", California Department of Food and Agriculture, Sacramento, Calif., 1974.
Plapp, F. W., Casida, J. E., *Anal. Chem.* **30**, 1622 (1958).
Safe, S., Hutzinger, O., Ed., "Mass Spectrometry of Pesticides and Pollutants", CRC Press, Inc., Cleveland, Ohio, 1973, pp 189–198.
Shafik, M. T., Bradway, D., Biros, F. J., Enos, H. F., *J. Agric. Food Chem.* 18, 1174 (1970).
Shafik, M. T., Bradway, D., Enos, H. F., *Bull. Environ. Contam. Toxicol.* **6**, 55 (1971a).
Shafik, M. T., Bradway, D., Enos, H. F., *J. Agric. Food Chem.* 19, 885 (1971b).
Shafik, M. T., Bradway, D. E., Enos, H. F., Yobs, A. R., *J. Agric. Food Chem.* 21, 625 (1973).
Shafik, M. T., Enos, H. F., *J. Agric. Food Chem.* **17**, 1186 (1969).
Spear, R. C., Jenkins, D. L., Milby, T. H., *Environ. Sci. Technol.* **9**(4), 308 (1975).
St. John, L. E., Lisk, D. J., *J. Agric. Food Chem.* **16**, 48 (1968).

Suzuki, K., Goto, S., Kashiwa, T., *Bunseki Kagaku* **17**(10), 1279 (1968).
Thompson, J. A., Markey, S. P., *Anal. Chem.* **47**, 1313 (1975).
Thompson, J. F., Ed., "Analysis of Pesticide Residues in Human and Environmental Samples", Section 6, Revised Dec 1974.
Van Wazer, J. R., Ed., "Phosphorus and Its Compounds", Interscience, New York, N.Y., 1961, p 1930.
Walker, W. W., Stojanovic, B. J., *J. Environ. Qual.* **2**, 474 (1973).
Wolfenden, R., Spence, G., *Biochim. Biophys. Acta* **132**, 296 (1967).
Yu, C. C., Sanborn, J. R., *Bull. Environ. Contam. Toxicol.* **13**, 543 (1975).
Zech, R., Wigand, K. D., *Experientia* **31**, 157 (1975).
Zuckerman, B. M., Deubert, K., Mackiewicz, M., Gunner, H., *Plant Soil* **33**, 273 (1970).

Received for review August 29, 1975. Accepted December 8, 1975. This research was supported in part by National Institutes of Health Trainee Grant No. ES00125, NIEHS.

In Vitro Inhibition of Lactate Dehydrogenase by Insecticidal Polychlorinated Hydrocarbons. I. Mechanism of Inhibition: Possible Association of Reduced Nicotinamide Adenine Dinucleotide with Mirex

Constance M. Hendrickson and Joe A. Bowden*

In order to aid in the clarification of the inhibitory mechanism operating in vitro on NADH-dependent dehydrogenases, we determined the effect of dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta-[*cd*]pentalene (Mirex) on the activity of crystalline rabbit muscle lactate dehydrogenase (EC 1.1.1.27, M_4 isozyme) at 340 nm. Mirex competitively inhibited with respect to both pyruvate and NADH (K_i values are 0.02 and 0.03 mM, respectively). A time-dependent association of Mirex with NADH was also observed as were changes in both uv and CD spectra. Based on the mechanism of lactate dehydrogenase (Holbrook, J. J., Gutfreund, H., *FEBS Lett.* **31**, 157 (1973)), we have postulated a mechanism for in vitro inhibition. This mechanism involves formation of a weak association complex between Mirex and NADH, and possibly some binding of Mirex with the enzyme molecule itself subsequent to formation of the Mirex–NADH–enzyme complex. The probable nature of the complex and implications of these findings are discussed.

Inhibition of lactate dehydrogenase and other NADH- or FADH-dependent enzymes by high molecular weight organochlorines possessing insecticidal activity has been observed by a number of groups (Abston and Yarbrough, 1974; Byczkowski, 1973; Gertig et al., 1970; Hendrickson and Bowden, 1973, 1975; McCorkle and Yarbrough, 1974; Freedland and McFarland, 1965). These inhibitions are of two types: in vitro inhibition of the enzyme itself (in cell-free systems) and in vivo inhibition, where enzyme activity is lowered after ingestion or injection of the compound. For example, the in vitro inhibition of lactate dehydrogenase by a number of chlorinated compounds appears to be the result of some direct (but unknown) effect on the enzyme molecule itself (Hendrickson and Bowden, 1973; Gertig et al., 1970). It is not known whether both types of inhibition are the result of the in vitro direct enzymatic effect, or whether separate reactions cause each type of inhibition. Even if enzymatic inhibition is not wholly responsible for the metabolic alterations observed in vivo (Byczkowski, 1973), it is of interest to study the mechanism by which this effect occurs, in order to delineate and clarify the various effects of these compounds on living systems at the cellular and subcellular levels, as well as on a gross physiological level. In a previous paper (Hendrickson and Bowden, 1975), we discussed the implications of inhibition of enzymatic activity by pesticides present at accumulation levels in affected species.

The compound under consideration, dodecachlorooctahydro-1,3,5-metheno-2*H*-cyclobuta[*cd*]pentalene (Mirex, shown in Figure 1) has been implicated in several instances of both in vitro and in vivo inhibition; among these are direct enzymatic inhibition of succinate dehydrogenase (McCorkle and Yarbrough, 1974) and of lactate dehydrogenase (Hendrickson and Bowden, 1973). In this paper, we have attempted to clarify the manner in which crystalline rabbit muscle lactate dehydrogenase (EC 1.1.1.27; M_4 isozyme) is inhibited by Mirex and have postulated a mechanism for this inhibition.

Department of Biochemistry, Louisiana State University, Baton Rouge, Louisiana 70803.

EXPERIMENTAL PROCEDURE

Materials. Crystalline rabbit muscle lactate dehydrogenase, sodium pyruvate, and NADH were obtained from Sigma Chemical Co. (St. Louis, Mo.). Analytical grade samples of Mirex (crystalline, 99+% purity) were supplied as a gift by Allied Chemical Corporation (Agricultural Division, Jackson, Miss.). All other compounds, solvents, and inhibitors were reagent grade, and water was carbon filtered and deionized.

Methods. *Determination of Initial Velocity.* Lactate dehydrogenase activity was assayed by following the rate of conversion of pyruvate to lactate in the presence of NADH by observing a change in absorbance of NADH at 340 nm with a recording Beckman DB spectrophotometer at 25 °C. Pyruvate and NADH solutions were prepared in 0.1 M potassium phosphate buffer at pH 7.5. NADH solutions were freshly prepared every 4 h. Mirex solutions were made up in 95% ethanol. Lactate dehydrogenase was diluted in 2.1 M ammonium sulfate (pH 6.0–6.1) to a final concentration of 10 IUB units per ml. Pyruvate was held constant at 1.3 mM and NADH varied from 0.005 to 0.36 mM, or NADH was held constant at 0.18 mM and pyruvate varied from 0.1 to 1.0 mM. NADH, pyruvate,