



## *Project Summary*

# Toxicology of Pesticides

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Documented in this report are the results of five toxicological studies of pesticide compounds conducted by the Institute for Medical Research and Occupational Health, Zagreb, Yugoslavia, for the U.S. Environmental Protection Agency.

In the first study, the reactions of two groups of esterases (cholinesterases and arylesterases) with substrates and inhibitors were investigated. Procedures for monitoring the absorption of phosalone and malathion in occupationally exposed workers by determination of pesticide residues in the urine were developed in the second study. This detection technique was compared to the traditional blood cholinesterase inhibition method to determine which was a more rapid detector of organophosphorus poisoning.

The third study surveyed the residues of chlorinated hydrocarbons in human milk and blood samples taken from the general population, and compared the observed levels with those found in the serum of workers exposed to pesticides. In the fourth study, cholinesterase activity was used to assess the effects of recent changes made in the protective procedures for occupationally exposed workers. Finally, in the last study, the alleged effect of pesticides on the eye and on vision was studied and the results discussed.

In addition, the report includes summaries of seven students' theses related to the work conducted by the Institute.

*This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a*

*separate report of the same title (see Project Report ordering information at back).*

### Introduction

Of all chemical compounds commercially used, pesticides pose one of the greatest and most direct threats to human health. To assure that all pesticides intended for widespread use are nonhazardous to humans, the U.S. Environmental Protection Agency's (EPA's) Health Effects Research Laboratory screens proposed compounds through extensive toxicity testing. The laboratory tests and analyzes compounds to provide the data necessary for EPA's regulatory activities, including the registration of new pesticides and suspension of pesticides proven hazardous after initial approval. The laboratory also develops and tests procedures for monitoring workers occupationally exposed to pesticides. Data gathered through such monitoring efforts are also used to support regulatory actions.

In support of the Health Effects Research Laboratory's testing and monitoring efforts, the Institute for Medical Research and Occupational Health (IMORH), Zagreb, Yugoslavia, has conducted five pesticide studies over a four-year period. This project summary presents and discusses the results of each of these studies. Only the major details of experimental procedures, however, are provided.

### Esterases and Organophosphorus Compounds

The focus of the first of the five studies conducted by IMORH was to determine the reactions of two groups of

esterases—cholinesterases and arylesterases—with their substrates and organophosphorus (OP) inhibitors.

### Procedure

Pyridinium oximes have long been recognized as effective reactivators of cholinesterases (ChE's) inhibited by OP compounds. It is their reactivating ability that makes them useful in the prevention and therapy of OP poisoning. In this study, 25 pyridinium oximes were synthesized in the laboratory and tested *in vitro* for the reactivation of phosphorylated and phosphonylated acetylcholinesterases (AChE's). The oximes were also tested *in vivo* as protective compounds against OP poisoning.

After initial testing, the binding sites for substrates and inhibitors of AChE and ChE were studied in detail. The kinetics of competition between pairs of substrates for AChE and ChE were investigated to determine whether or not the substrate-inhibition site takes part in the reaction.

In addition, ChE's and arylesterases were tested comparatively in several species using O,O-dimethyl-2,2-dichlorovinylphosphate (DDVP) as ChE inhibitor and arylesterase substrate.

### Results

Sixteen of the 25 newly synthesized compounds were tested for their ability to reactivate human erythrocyte methyl-ethoxyphosphonylated AChE. Of these 16 oximes, three proved to be fairly good reactivators. The reaction of these three compounds was initially rapid, but they slowly tapered down to an equilibrium. Although all three compounds had a similar reactivation efficiency, none had better reactivation properties than TMB-4, a well-known reactivator of OP-inhibited AChE which was tested in the study as a reference compound.

The same 16 compounds discussed above were also investigated to determine their protective characteristics against AChE inhibition by O-1,2,2-trimethylpropylphosphonofluoridate (Soman). Protective efficiency was evaluated by comparing AChE inhibition by Soman, with and without the tested compound. Benzolcarbonyl, cyclohexylcarbonyl, and amidocarbonyl exerted a good protective effect against AChE inhibition by Soman; TMB-4 offered no protection from Soman inhibition.

The nine other oximes (bispyridinium) were tested for reactivation potency and therapeutic effect on two OP com-

pounds, DDVP and O-ethyl-S-2-diisopropylaminoethyl methylphosphonothioate (VX). Enzyme reactivation was measured on human erythrocyte AChE, and therapeutic effect was evaluated on male albino rats. The oximes with a hydroxyamino group in position 4 in the pyridinium ring were good reactivators of both phosphorylated and phosphonylated AChE. The same oximes were also effective (given with atropine) against VX and DDVP poisoning.

To determine whether or not the substrate-inhibitor site in an enzyme takes part in the reaction, the binding sites in bovine erythrocyte AChE were studied in the presence of several different inhibitors. After a series of kinetic experiments investigating paired substrate competition for erythrocyte AChE and horse serum ChE, it was concluded that the substrate-inhibition site was not involved in the competition between two substrates. However, when the OP compound haloxon was the inhibitor, the reaction did occur near the site.

To determine whether regeneration *in vivo* was spontaneous or was due to enzyme synthesis, comparative studies of the reaction of ChE's and arylesterases with DDVP were conducted in various species. Pure DDVP and DDVP derived from metrifonate were used as the ChE inhibitor and arylesterase substrate. Kinetic analysis of *in vivo* rat brain and plasma data showed that regeneration of enzyme activities after DDVP treatment could be attributed entirely to spontaneous activation of the inhibited enzymes; no difference was seen in the kinetics of the reaction in different species, and the activity of the arylesterases was of the same order in mammalian and nonvertebrate tissues. Regeneration of ChE activity in human plasma and erythrocytes *in vivo* was attributed to enzyme synthesis, but since 6 h elapsed before ChE determination, the enzyme had reached an aged, non-reactable form.

### Residues of OP Pesticides in Human Urine

In the second study, IMORH researchers compared two methods—the blood ChE inhibition method and the urine pesticide residues method—to establish which of the two was a more reliable indicator of the amount of OP pesticides absorbed by occupationally exposed workers. Urinary metabolite analysis was hypothesized to detect the

absorption of OP pesticides prior to any depression of ChE activity, thus permitting protective measures to be taken sooner than allowed by the standard ChE inhibition method.

### Procedure

To evaluate this hypothesis, the effects of phosalone, malathion, and quinalphos on occupationally exposed workers were investigated by analyzing urine samples obtained after exposure and by measuring blood ChE inhibition levels.

Urine samples from 12 workers exposed to phosalone and 14 workers exposed to malathion were analyzed for their alkali metal salts concentration. These salts result from the hydrolysis and metabolism of the dialkyl esters of the acids produced during OP pesticide degradation. Determination of the amounts of these salts in urine and blood samples can be used to detect OP poisoning. Simulated samples were prepared for purposes of comparison by adding a definite amount of standard salt to the urine of nonexposed persons.

In addition, a volunteer subject with no previous exposure to phosalone was experimentally exposed for the duration of one working day, and then again on the three following days. Urine samples were taken at the beginning and the end of work, and 4 to 5 h after termination of exposure. Using the data obtained from this experiment, the period necessary for the complete excretion of pesticide residues and the appropriate time for urine sampling were determined.

Analysis of samples obtained from workers exposed to phosalone and malathion consisted of measuring the concentration of O,O-diethyl phosphorodithioate (DEDTPK) and O,O-dimethyl phosphorodithioate (DMDTPK) alkali metal salts in the urine. Various diazoalkanes were applied to convert the salts into more volatile trialkyl derivatives for gas chromatography (GC) analysis. Urine samples from malathion-exposed workers were collected at the end of work hours. Urine samples from phosalone-exposed workers were collected both prior to the beginning of work and at the end of work.

The absorption of quinalphos by 11 exposed workers from a pesticide production plant was studied by determining the amount of O,O-diethyl phosphate (DEP) and O,O-diethyl phosphorothionate potassium salt (DETPK) in the workers'

urine. Urine samples were collected at the start and end of work at intervals of 30 days during three months and immediately after one month's vacation. Blood samples were taken at the beginning of work on the same days as urine samples were taken. Eleven urine samples were also collected from non-exposed workers and analyzed for comparison.

## Results

DEDTPK was found in the urine of all 12 production workers exposed to phosalone. Only the two highest values measured in the urine samples were also detected by the blood ChE inhibition method, supporting the hypothesis set forth at the beginning of the study.

DMDTPK concentrations found in 10 of 14 urine samples obtained from workers exposed to malathion fell within the range of 449 to 1072 ng/ml of urine. In remaining urine samples, and in nine samples from non-exposed persons, no malathion residues were detected. The highest concentration of malathion residue in urine was accompanied by the lowest ChE activity.

Results from the experimental exposure of a volunteer to phosalone showed that the amount of residues excreted increased gradually, and the highest value was reached 4 to 5 h after exposure. Subsequently, the amount of residue decreased abruptly, but was still measurable at the beginning of the following work day. Residues increased systematically when exposure was prolonged for several days. The volunteer's blood and plasma ChE was only slightly reduced during exposure.

The total amount of all metabolites excreted during one day was determined a better indicator of exposure than metabolite concentration in a single urine fraction. However, because of the latter technique's inconvenience, the concentrations of DEDTPK taken for the same period of time after exposure can be used instead.

Results from the study conducted to compare the effect of quinalphos on blood ChE inhibition and urine residues concentration showed that the highest concentration of residues in urine was followed by the greatest decrease of ChE activity. However, since the workers tested alternated three to five days between work with OP compounds and other compounds such as carbamates, no determination of one method's desirability over the other could be made.

## Residues of Chlorinated Hydrocarbons in Human Milk and Blood

The third IMORH study was conducted to determine the amount of chlorinated hydrocarbon (HC) residues in serum samples taken from two groups of occupationally exposed workers and from the general population of four different parts of Yugoslavia. In addition, mother's serum, mother's milk, and umbilical cord serum samples taken between 1977 and 1979 in the Yugoslavian town of Croatia were analyzed for DDT content.

## Procedures

Pesticide residue levels in 262 blood serum samples taken from members of the general population and in 78 serum samples taken from exposed workers were determined by GC. Samples were analyzed for p,p'-DDT, p,p'-DDE, and p,p'-DDD, Lindane, and  $\alpha$ -HCH.

Residue levels in mother's serum, mother's milk, and cord blood serum were also determined by GC. Concentrations of p,p'-DDE, p,p'-DDD, and p,p'-DDT were measured in 34 human milk samples collected three to five days after delivery and in 37 samples collected 1 1/2 to 55 weeks after delivery. Two extraction methods for milk were used and compared.

In the first method, 1 ml milk was extracted with acetonitrile and the extracts combined with sodium sulfate. This mixture was then extracted with hexane, and the hexane extracts purified on a florisil column. Organochlorine compounds retained on the column were eluted with hexane, and the eluates evaporated to dryness in a nitrogen stream. The compounds were redissolved for GC analysis.

In the second method, 0.5 ml milk was partitioned with methanol and potassium carbonate. The mixture was extracted with hexane, and the hexane extracts evaporated to dryness. The compounds were redissolved in hexane for GC analysis.

For extracting residues from the mother's and cord serum samples, serum was partitioned with formic acid and the resulting mixture extracted with hexane. The hexane extracts were washed and purified on a florisil column. Compounds retained on the column were eluted with hexane, and the eluates evaporated to dryness in a nitrogen stream. The compounds were redissolved in hexane and analyzed.

## Results and Discussion

Concentrations of DDT and hexachlorocyclohexane residues found in the blood serum of the general population fell within the range reported for other countries. Few samples contained Lindane or  $\alpha$ -HCH, but the mean concentrations for these compounds were much lower for the general population than for occupationally exposed workers. Exposed workers also had a higher incidence of residues in the serum.

Differences in the mean concentration values for p,p'-DDE measured in milk samples obtained at the beginning of lactation and samples obtained during the subsequent 55 weeks were not found to be significant. The two extraction methods for milk compared favorably for p,p'-DDE. The content of DDT derivatives in milk fell within the middle of the range for European and non-European countries.

Concentrations in mothers' milk were 2.1 times higher than in mothers' serum. Cord blood serum contained lower concentrations of p,p'-DDE than the mother's serum. Serum samples from non-pregnant women had the same DDT content as those from mothers at delivery.

## Biochemical and Clinical Effects of Pesticides in Humans

The IMORH's fourth study attempted to relate clinical symptoms in workers to the degree of their exposure to a wide variety of pesticides. Vitamin A levels and DDT concentrations in exposed workers were measured and compared to values obtained for non-exposed persons.

## Procedures

To evaluate the absorption of pesticides by 567 industrial workers during the period from 1970 to 1979, measurements of blood ChE were taken regularly. Only 170 of the 567 workers studied worked at any of three production lines (dust or wettable powder, emulsion, or household sprays) in one plant for 2 to 14 years. The others were seasonal workers hired for short periods of intense production.

In the nine-year study period, workers were exposed to OP compounds, carbamate insecticides, herbicides, fungicides, and other compounds. Plasma and erythrocyte ChE activity were determined by spectrophotometry.

Vitamin A levels in the serum of 65 exposed workers were measured in

1976, and again in 1977 for some. Vitamin A levels for the control group were measured in both 1976 and 1977. Vitamin A was determined by spectrophotometry with antimony trichloride.

Total DDT in the serum and ChE activity in the blood and plasma of exposed workers were also measured to verify that pesticides were absorbed. Since exposure to certain pesticides was not continuous, ChE activity was monitored both before the beginning of work with AChE pesticides and during the course of work with these pesticides.

## Results

Since the production of OP insecticides increased during the 10-year observation period, the number of blood samples with decreased enzyme activity was also expected to increase. However, the greatest depression of enzyme activity below 50% of normal occurred in the first two years, when workers were exposed to the extremely toxic insecticides dimethoate and chlorfenvinphos. Once this was noted working conditions were improved, resulting in no cases of poisoning in 1972 and only two in 1973. Enzyme depression below 50% again rose in the following years when production of insecticides increased and more inexperienced workers were hired.

Results of measurements of Vitamin A showed no effect of pesticides on Vitamin A levels. All control and exposed group Vitamin A levels fell within the same range along with levels measured for standards.

Measurements of ChE activity made before the initiation of work with AChE pesticides showed that ChE activity was not reduced more than 20% in any of the groups. During the course of work with pesticides the same workers showed greatly reduced ChE activity levels, indicating that the workers had absorbed quantities of the pesticides.

Workers exposed to chlorinated HC's had higher serum DDT levels than controls. DDT levels found in workers not exposed to chlorinated HC's were comparable to those found in the general population earlier.

## Conclusions

When protective devices and sanitation regulations were enforced after the first two years, the absorption of AChE insecticides was satisfactorily reduced. In addition, weekly measurement of ChE

activity was shown to be a practical method for determining worker risk and thus allowing the prevention of further pesticide absorption.

## Effect of Pesticides on the Eye and Vision

The last study reported by IMORH was conducted to investigate the effects of pesticides on the human eye and vision.

## Procedures

Fifty-seven of the permanent workers exposed to AChE pesticides in the previous study (47 production and 10 pesticide application workers) were selected for this investigation. Eighteen of the workers were exposed from one to five years, and the other 39 were exposed for over five years. Only 11 were older than 45. A detailed history of illnesses possibly connected with eyesight was gathered for each worker, and all workers underwent ophthalmological examinations, including tonometry, ophthalmoscopy, slit-lamp biomicroscopy, keratometry, and visual acuity tests. Peripheral vision was measured by a Goldmann perimeter, and dark adaptation ability was assessed on a Goldmann-Weekers adaptometer. Workers with visual abnormalities were treated by standard procedures.

## Results

Twenty-three of the production workers complained of lacrimation, photophobia, itching and burning, and other maladies. Only three of the application (agricultural and public health) workers complained of these eye problems.

The most frequently observed abnormality of the frontal eye segment was dilated or tortuous episcleral blood vessels. The incidence of these abnormalities was much higher in the exposed workers than in the same-age members of the general population. Limited conjunctival injection, abnormal pupils, and lens opacities were also noted in some workers. Four production and one application worker had pronounced astigmatisms. Keratometric measurements showed little abnormality in the majority of the workers, however.

Increased intraocular pressure was noted in six workers; open angle glaucoma was subsequently diagnosed in one of the six, but the phenomenon remains unexplained for the other five. Also unexplained is the constriction of the visual field observed in 11 production and 2 application workers. This was

the most common of all abnormalities observed in the workers tested.

Dark adaptability was measured as remarkably slow in 2 of 16 production workers tested, but no or little difference was noticed in the other 14 workers.

## Appended Students' Theses

The seven students' theses summarized in an appendix to the report include the following titles:

- Preparation of Silyl Derivatives for the Gas Chromatographic Analysis of Organophosphorus Pesticide Residues
- Simultaneous Gas Chromatographic Determination of Alkali Metal Salts of O,O-Diethyl-, O,O-Dimethyldithio-, and O,O-Diethylthio-phosphoric Acid.
- Toxic Effects of Metrifonate in Mammals
- Mechanism of Inhibition of Acetylcholinesterase by Some Oximes
- Determination of Organophosphorus Pesticide Residues by Gas Chromatography
- Organophosphorus Pesticides in Surface Waters

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The complete report, entitled "Toxicology of Pesticides," (Order No. PB 82-226 077; Cost: \$9.00, subject to change) will be available only from:

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