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TOXICOLOGY OF PESTICIDES

by

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16. ABSTRACT

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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 poisoning.

The third study reported surveyed the residues of chlorinated hydrocarbons in human milk and blood samples taken from the general population, and compared the 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 were studied and the results discussed.

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

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony adn preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

Of all chemical pollutants, pesticides pose one of the most direct threats to human health. Thus, to assure the safety of pesticides before their commercial marketing, researchers must investigate their effects through extensive toxicological testing. In addition to laboratory testing and analysis of pesticide compounds, these researchers must also develop effective monitoring techniques for determining the safety of workers handling pesticides.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

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 poisoning.

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SECTION 1

INTRODUCTION

This is the final report of our four year study of the toxicology of pesticides. It consists of six sections, each written as an independent entity. The report also includes summaries of seven students's these related to the work. The theses were supervised by senior members of our institute, which is also a teaching institution of the University of Zagreb. The references include our publications since 1976.

Most studies described in this report are a continuation of work started before 1976. Studies were initiated by EPA and are now included in the Institute's research program. Studies described under section 4 are part of the World Health Organization/United Nations Environmental Planning (WHO/UNEP) "Pilot project on assessment of human exposure to pollutants through biological monitoring". The Institute is a "Collaboratong Center for Pesticides Toxicology" of WHO.

SECTION 2

ESTERASES AND ORGANOPHOSPHORUS (OP) COMPOUNDS

Reactions of two groups of esterases, the cholinesterases (ChEs) (EC 3.1.1.7 and EC 3.1.1.8) and the arylesterases (EC 3.1, 1.2), with substrates and inhibitors were studied.

In addition to this report, we have published these results in nine papers. (Reiner 1980, Reiner et al. 1977b, 1978a,b, 1979a, 1980, Simeon et al. 1977, 1979, Skringove -Spoljar et al. 1980), seven communications (Reiner 1977a, Reiner et al. 1976, 1979b, Simeon et al. 1978a, b, Skrenjaric-Spoljar et al. 1978, 1979) and one thesis (Radic 1979). Details of experimental procedures and references to the literature may be found in these publications.

Material and Methods

The following enzyme preparations were used: purified bovine erythrocyte acethylcholinesterase (AChE), purified horse serum ChE, native plasma and erythrocytes from man, rabbit, rat, hamster and chicken, liver and brain homogenates from rat and hamster, and homogenates from different parasitic helminths.

The enzyme activities were determined either by spectrophotometric or manometric methods. (Ellman et al. 1961, Krupka 1966, Reiner et al. 1978). Partial purification of arylesterases from human erythrocytes was done by gel filtration.

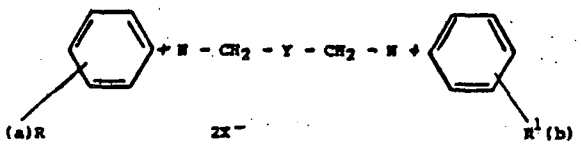
RESULTS AND DISCUSSION

Reactivating and Protective Effects of Pyridinium Compounds in AChE Inhibition by OP

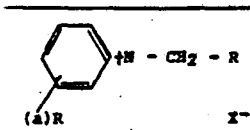
It is well known that pyridinium oximes are effective reactivators of ChEs inhibited by OP compounds; these oximes are also effective in protection against and therapy for OP poisoning (SIPRI 1976). More recently, pyridinium compounds with no oxime group have also shown some activity *in vivo*.

The reactivation of human erythrocyte AChE (EC 3.1.1.7) inhibited by O-ethyl-S-2-diisopropylaminoethyl methylphosphonothioate (VX) and the protection against AChE inhibition by O-1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) were studied with sixteen quarternized pyridinium compounds. Table 1 gives the structural formulae of these compounds, the dissociation constants of the AChE - reactivator complex in the presence of 1.0 mM acetylthiocholine (Ki(app)) and rate constants for 1.0 mM acetylthiocholine hydrolysis in the presence of pyridium compounds (k').

TABLE 1. REACTIVATION OF AChE* BY 16 PYRIDINIUM COMPOUNDS



Abbreviation	R	(a)	Y	R^1	(b)	X	K_i(app) (mM)	k^1 (M^{-1} min^{-1})
I	CH = NOH	4	CH_2	CH_3	4	Br	0.06	-
II	CH = NOH	4	CH_2	$CONH(CH_2)_3OH$	3	Br	0.08	31
III	CH = NOH	4	CH_2	$CONH(CH_2)_3OH$	4	Br	0.27	38
IV	CH = NOH	4	CH_2	-	-	Br	0.23	35
V	CH_3	2	CO	$CONH_2$	3	Br	0.04	10
VI	CH_3	2	CO	CH_3	2	Br	0.26	7
VII	CH = NOH	4	CO	CH_3	2	Br	0.02	31
VIII	CH = NOH	4	CO	CH_3	4	Br	0.06	26
HGG-12	CH = NOH	2	0	COC_6H_5	3	J	0.11	37
HGG-42	CH = NOH	2	0	COC_6H_{11}	3	J	0.18	30
HI-6	CH = NOH	2	0	$CONH_2$	4	Cl	0.16	22
TMB-4	CH = NOH	4	CH_2	CH = NOH	4	Br	1.0	69



Abbreviation	R	(a)	R	X	K_i(app) (mM)	k^1 (M min^{-1})
IX	CH = NOH	4	$(CH_2)_2BR$	Br	0.11	34
X	CH = NOH	4	$(CH_2)_2$-N⟨⟩	Br	0.07	26
XI		-	$COCH_2BR$	ClO_4	0.20	-
XII		-	$COCH_2BR$	Br	0.05	-

*Inhibited by UX and Soman

TMB-4, which is known as a good reactivator of AChE inhibited by OPs proved to be the most effective reactivator. Of the newly synthetized compounds tested, three were fairly good reactivators of methylethoxyphosphonylated AchE. These compounds have two pyridinium rings connected by a dimethylether link and a hydroxyiminomethyl group on position 2 of one pyridinium ring, while the radicals of the other pyridinium ring are benzoylcarbonyl (HGG-12), cyclohexylcarbonyl (HGG-42) or amidocarbonyl (HI-6) residue.

The rate of reactivation with these compounds followed a two-phase pattern, being fast at the beginning and then slowing down to an equilibrium. Kinetic treatment of the first-phase reaction course yielded the second-order rate constants of reactivation (k_r) (Table 2). All three compounds had

TABLE 2. REACTIVATION OF METHYLETHOXYPHOSPHONYLATED AChE BY BISPYRIDINIUM ALDOXIMES

Compound	Evaluated Constants*			Protective Index
	k_{+2} (min^{-1})	K_r (uM)	$k_r \cdot 10^{-3}$ (M^{-1} min^{-1})	
HGG-42	0.04	50	0.8	2.51
HGG-12	0.06	45	1.4	3.27
HI-6	0.09	26	3.6	3.99
TMB-4	0.03	2	19.4	NII

*The above constants refer to the following reaction:

$$\text{Phosphorylated AChE} + \text{Oxime} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{Complex} \xrightarrow{k_{+2}} \text{Free AChE} + \text{Phosphorylated oxime}$$

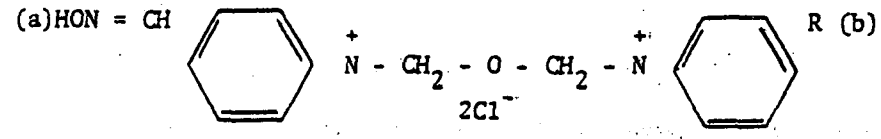
where K_r equals: $\dfrac{k_{+2} + k_{-1}}{k_{+1}}$

similar reactivating efficiency (k_r values range from 0.8×10^3 to 3.6×10^3 $M^{-1}min^{-1}$) but in effective concentrations (1 to 100 µM) they also inhibited AChE ($K_{i\ (app)}$ values range from 0.11 to 0.19 mM). Their reactivating properties were not better than those revealed by TMB-4 ($k_r = 19.4 \times 10^3$ M^{-1}) which was tested as a reference compound.

Protective efficiency was evaluated by comparing AChE inhibition by Soman; with and without the tested compound (0.1 mM final). The time course of inhibition by Soman was measured, and log percent activity was plotted as a function of time. The first-order rate constants of AChE inhibition by Soman were evaluated from the linear part of the inhibition curves with and without the addition of the pyridinium compound. As a measure of protective efficiency, quotients of the rate constant without and the rate constant with 0.1 mM pyridinium compound present were calculated (protective index). HGG-12, HGG-42 and HI-6 were also found to exert a good protective effect against AChE inhibition by Soman; no protection was obtained with TMB-4 (Table 2).

In addition, nine other bispyridinium oximes were synthesized and tested for reactivating potency and therapeutic effect on two OPs: O,O-dimethyl-2, 2-dichlorovinylphosphate (DDVP), and VX. The reactivation was measured on human erythrocyte AChE and the therapeutic effect was evaluated on male albino rats. Tested compounds contained two pyridinium rings linked by dimethylether; each compound had a hydroxyiminomethyl group on position 2 or 4, on one of the pyridinium rings, while the other ring was unsubstituted or had a methyl or a dydroxyiminomethyl group in position 2 or 4 (Table 3). The oximes with a

TABLE 3. REACTIVATION OF OP INHIBITED AChE BY OXIMES



Abbreviation	(a)	R	(b)	pk_1	pk_2	$k_r 10^{-3}/M^{-1}$ VX	mim^{-1} DDVP
HI_4	(4)	-H	(-)	7.7	-	4.1	13
4.2-MEDP	(4)	$-CH_3$	(2)	7.9	-	6.3	6.2
4.4-MEDP	(4)	$-CH_3$	(4)	7.8	-	4.3	*
Toxogonin	(4)	-CH = NOH	(4)	7.5	8.6	6.6	14
HS-3	(4)	-CH = NOH	(2)	7.8	8.4	8.6	4.8
HS-14	(2)	-H	(-)	7.4	-	0.34	*
2.2-MEDP	(2)	$-CH_3$	(2)	-	-	-	*
2.4-MEDP	(2)	$-CH_3$	(4)	7.6	-	*	*
HS-4	(2)	-CH = NOH	(2)	6.7	7.7	*	0.07
PAM-2	$C_5H_4N-CH_3$ $[_2CH = NOH]Cl$			7.8		1.2	2.5

*No reactivation was observed.

hydroxyimino group in position 4 in the pyridinium ring were good reactivators of both phosphorylated and phosphonylated AChE. The same oximes were also very effective given with atropine against VX and DDVP poisoning (Table 2). The compounds are almost as effective as PAM-2, but PAM-2 is less toxic.

TABLE 4. EFFICIENCY OF OXIMES AND ATROPINE AGAINST VX AND DDVP POISONING*

Antidotes	Toxicity LD50/mg kg^{-1}		Relative efficiency Against VX and DDVP		
			DDVP	DDVP	VX
	i.v.	i.p.	(p.o.)	(s.c.)	(s.c.)
Atropine (alone)	-	-	7.7	12	4.6
Oxime + atropine					
HI-4	47	56	8.8	-	43
4,2-MEDP	59	59	7.0	37	47
4,4-MEDP	100	119	8.4	34	47
Toxogonin	100	200	8.4	34	40
HS-3	168	149	7.0	34	89
HS-14	66	-	9.9	43	26
2,2-MEDP	54	-	6.5	-	-
2,4-MEDP	170	178	-	-	28
HS-4	67	168	6.5	-	-
PAM-2	101	212	8.4	24	47

*
**(10 mg/kg)
i.v. = intravenous
i.p. = intraperitoneal
p.o. = by mouth
s.c. = subcutaneous

Binding Sites for Substrates and Inhibitors in AChE and Cholinesterase

It is known that certain substrates act also as inhibitors of the cholinesterases. The mechanism of this reaction is complex and not fully understood (Aldridge et al. 1972, Reiner 1975). Studies of the substrate-inhibition-site are described in this section.

AChE (EC 3.1.1.7) from bovine erythrocytes is inhibited by haloxon (di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate), both irreversibly and reversibly (temperature range 5 °C-40 °C).

$$\text{AChE} + \text{Haloxon} \underset{}{\overset{K_I}{\rightleftharpoons}} \text{Reversible complex} \overset{K_a}{\longrightarrow} \text{Phosphorylated enzyme}$$

The second-order rate constants of irreversible inhibition (k_a) increase with temperature, and the activation energy is 84 kJ mol^{-1} (Table 2). The dissociation constants of the enzyme-inhibitor reversible complex (K_I) range from

3.6 µM (at 5°C) to 6.5 µM (at 40°C). The dissociation constants of the enzyme-acetythiocholine complex, K(s), derived from the reversible inhibition experiments range from 2.5 mM (at 5°C) to 4.1 mM (at 40°C) (Table 5). These

TABLE 5. EFFECT OF TEMPERATURE ON INHIBITION OF AChE BY HALOXON

Temp. °C	10^{-3} k_a M^{-1} s^{-1}	K_I µM	N*	K(S) mM
5	0.165	3.6	28	2.5
11	0.377	4.2	12	1.9
18	0.656	5.5	3	2.1
25	2.08	6.4	87	2.3
32	3.97	9.1	3	7.7
40	9.08	6.5	7	4.1

*N = Number of experiments

constants are considerably larger than the Michaelis constant (Km) for AChE and acetylthiocholine, and agree more closely with the substrate inhibition constant (K_{ss}) for acetylthiocholine, indicating that the competition between haloxon and acetylthiocholine occurs close to the substrate-inhibition site.

The kinetics of competition of pairs of two substrates for bovine erythrocyte AChE (EC 3.1.1.7) and horse serum ChE (EC 3.1.1.8) were studied in such way that the hydrolysis of only one substrate was measured at a time. The substrates were acetylthiocholine, phenylacetate and benzoylcholine; the same compounds, and also acetylcholine, were used as competing substrates i.e. inhibitors. The substrate inhibition constants (K_{ss}) and Michaelis constants for the reaction of a single substrate were also determined.

In Table 6, the K_m and K_{ss} values are given, the dissociation constants for the enzyme-substrate (K(S)) and enzyme-inhibitor (K(I) complexes are listed in Table 7. Comparing these sets of data, it was concluded that the substrate-inhibition-site in the enzyme does not show up in the competition between two substrates.

Reaction of DDVP and O,O-Dimethyl-2,2,2-Trichloro-1-Hydroxyethyl-Phosphonate and (Metrifonate) with Cholinesterases and Arylesterases of Various Species

Metrifonate (also called trichlorfon) and DDVP are widely studied compounds because metrifonate is a drug used against schistosomiasis and DDVP is a well known pesticide (Holmstedt et al. 1978, Nordgren et al. 1978, Wright et al. 1979). Furthermore, metrifonate rearranges spontaneously into DDVP and all reactions of metrifonate are therefore accompanied by simultaneously proceeding reactions of DDVP. It is known that mammalian cholinesterases (EC

TABLE 6. MICHAELIS CONSTANTS AND SUBSTRATE INHIBITION CONSTANTS FOR AChE AND ChE

Substrate (mM) (Range of Conc.)	K_m/mM	K_{ss}/mM
Acetylcholinesterase		
Acetylcholine (0.01-1.0)	0.15	-
Acetylcholine (1.0-100)	-	9
Acetylthiocholine (0.1-50)	0.11	14
Phenylacetate (0.1-10)	2.6	-
Cholinesterase		
Benzoylcholine (0.01-10)	0.4(n_H* = 0.7)	-
Benzoylcholine (10-100)	-	56
Acetylcholine (0.1-10)	1.2-3.2	-
Acetylthiocholine (0.1-10)	0.6(n_H = 0.8)	-
Phenylacetate (0.1-10)	3.8	-

*nH = Hill coefficient

TABLE 7. COMPETITION BETWEEN TWO SUBSTRATES FOR AChE AND ChE

Substrate	K(S)mM	Inhibitor (mM)(Range)	K(I)mM
Acetylcholinesterase			
Acetylthiocholine	0.17 ± 0.17	Acetylcholine (0.5-10)	0.22 ± 0.22
Acetylthiocholine	0.22 ± 0.14	Phenylacetate (2.6-15)	4.9 ± 6.1
Phenylacetate	3.8 ± 0.8	Acetylthiocholine (0.2-5)	0.25 ± 0.03
Phenylacetate	6.2 ± 1.2	Acetylcholine (0.5-5)	0.54 ± 0.05
Cholinesterase			
Acetylthiocholine	0.53 ± 0.17	Acetylcholine (5-20)	2.6 ± 0.77
Acetylthiocholine	≈1.0	Phenylacetate (1-10)	≈4.0
Acetylthiocholine	0.37 ± 0.55	Benzoylcholine (0.05-1)	0.03 ± 0.04
Phenylacetate	2.5 ± 0.4	Acetylthiocholine (0.2-4)	0.69 ± 0.08
Phenylacetate	1.6 ± 0.4	Acetylcholine (5-15)	3.1 ± 0.7
Benzoylcholine	0.17 ± 0.45	Acetylthiocholine (10-50)	1.3 ± 3.3

3.1.1.7 and 3.1.1.8) are inhibited by DDVP. The rates of the *in vitro* inhibition, spontaneous reactivation, and aging of the inhibited enzymes are also known. A calculation is presented here in which rate constants determined *in vitro* are applied to enzyme activities measured *in vivo*. The purpose was to discover whether regeneration *in vivo* is due to spontaneous reactivation of the inhibited enzyme or to enzyme synthesis. Two groups of data are used, one for human blood ChE's and the other for rat brain and plasma ChE's. Two OP compounds were studied, DDVP, and metrifonate. The latter is not a ChE inhibitor, but, in aqueous solutions it rearranges spontaneously into DDVP, which is an inhibitor of the enzyme.

The data for the *in vivo* regeneration of rat brain and plasma cholinesterases are given in Table 8. The kinetic analysis of the data has proved

TABLE 8. BRAIN AND PLASMA ChE ACTIVITIES OF RATS INJECTED I.V. WITH METRIFONATE OR DDVP*

Time after dosing	Cholinesterase activity (%)			
	Metrifonate 300 mg/kg		DDVP 2.5 mg/kg	
	Brain	Plasma	Brain	Plasma
30 min	2	7	15	41
60 min	6	7	30	44
90 min	13	14	36	59
2 h	14	19	50	74
3 h	35	33	56	93
6 h	68	73	85	94
12 h	68	74	83	100
24 h	84	95	87	103
2 days	78	93	90	-
6 days	83	95	82	-
8 days	77	102	82	-

*The activities are expressed as percentage activity of untreated animals. Each value is the mean value obtained in 5 to 12 animals. The number of untreated rats was 40.

that regeneration of the enzyme activities after treatment with DDVP can be attributed entirely to spontaneous reactivation of the inhibited enzymes, with half-times of 2 and 2.5 h for the brain and plasma ChEs, respectively. Table 9 gives data for the *in vivo* regeneration of human plasma and erythrocyte ChEs after treatment with metrifonate. The first determination of ChE activities was done 6 h after dosing. It follows from the *in vitro* results that after that time almost all enzyme is present in a non-reactable (aged) form. Regeneration of the ChE activities (which was much slower than predicted from

TABLE 9. ERYTHROCYTE AND PLASMA ChE ACTIVITIES* IN SCHOOLCHILDREN TREATED ORALLY WITH METRIFONATE

Time after dosing	Cholinesterase activity (%)					
	Group A (21) 7.5 mg/kg Metrifonate		Group B (19)		Group C (19)	
	Erythrocyte	Plasma	Erythrocyte	Plasma	Erythrocyte	Plasma
6 hr	78	5	43	0	59	0
24 hr	87	19	51	17	60	14
3 days	72	28	63	35	76	31
7 days	80	64	65	47	62	56
14 days	92	76	72	71	80	72

*The activities are expressed as percentage activity before treatment, and each number is the mean value obtained for the group.

spontaneous reactivation) was therefore attributed to enzyme synthesis. The calculated half-times for the synthesis of human erythrocyte and plasma ChEs are 15 and 6.7 days, respectively.

The reaction of DDVP with ChEs in parasitic helminths was studied, because DDVP formed from metrifonate might be the active compound of the antiparasitic drug.

The cholinsterase of the nematode parasite Metastrongylus apri (M. apri) was studied at 25°C in 0.1 M phosphate buffer pH 7.4. The activity vs substrate concentration curves for acetylthiocholine (ATCh) and butyrylthiocholine (BTCh) are both bell-shaped, and each has an optimum at 10mM substrate. The Michaelis constants for ATCh and BTCh are 0.1 and 0.8 mM, respectively. The substrate inhibition constants for ATCh and BTCh are >100 and >>30 mN, respectively. At optimum substrate concentration, ATCh is hyrolyzed 15 times faster than BTCh. (Table 10). The enzyme is soluble (97%) in 0.15 M NaCl. DDVP and haloxon are progressive inhibitors with rate constant of inhibition 7.2×10^4 and 2.5×10^5 $M^{-1}min^{-1}$, respectively. Metrifonate is not an inhibitor. The results indicate that M. apri has only one ChE, but it could not be established whether it is the E.C. 3.1.1.7 or E.C. 3.1.1.8 enzyme.

DDVP was used to establish the number of ChEs in certain species. The protein fractions, soluble in 0.15 M NaCl, of Paramphistomum microbothrium, Ascaris suum and Neoascaris vitulorum each contain at least two ChEs. This is deduced from the degree of inhibition by DDVP which is different when measured with ATCh than with BTCh as substrate (Table 11). As Table 12 shows, the ChEs of these species are only partially soluble in saline.

TABLE 10. KINETIC CONSTANTS FOR THE REACTION OF ChE WITH ATCh AND BTCh

Enzyme	K_m/mM	K_{ss}/mM	$\frac{v \text{ for ATCh}}{v \text{ for BTCh}}$
*Metastrongylus apri ChE			
ATCh	0.1	>100	15
BTCh	0.8	>> 30	
Erythrocyte ChE			
ATCh	0.11	14	107
BTCh	0.42	30	
Serum ChE			
ATCh	0.60	-	0.46
BTCh	0.41	-	

*The activities for the M. apri enzyme were measured at nine different concentrations of ATCh and seven different concentrations of BTCh, in enzyme preparations from five and eight batches of parasites respectively.

TABLE 11. INHIBITION OF ChE* BY DDVP IN PARASITE SPECIES

Parasite species	Conc. Time	Inhibition (%) determined with ATCh	(N)	BTCh	(N)
Paramphistomum microbothrium	10 µM DDVP				
	5 min	44	(4)	6	(2)
	10 min	64	(19)	31	(18)
Ascaris suum	2.0 µM DDVP				
	3 min	74	(8)	26	(3)
	5 min	82	(8)	28	(7)
Ascaris suum anterior part	2.0 µM DDVP				
	1 min	51	(6)	17	(5)
	2 min	57	(6)	23	(5)
	3 min	58	(5)	36	(5)
	4 min	67	(4)	48	(5)
Neoascaris vitulorum	2.0 µM DDVP				
	1 min	25	(5)	Zero	(4)
	3 min	45	(4)	27	(3)
	5 min	57	(3)	19	(4)

*At 25°C and pH 7.4
N = number of assays

TABLE 12. ChE ACTIVITIES* IN PARAMPHISTOMUM MICROROTHRIUM AND ASCARIS SUUM FOR 10 mM ATCh AND 10 mM BTCh

	P. microbothrium	A. suum
Activity in homogenates against ATCh	$\mu mol\ h^{-1} g^{-1}$	
Mean (n)	47 (6)	43 (7)
Range	35-62	34-58
Activity ratio ATCh/BTCh in homogenates	Ratio	
Mean (n)	1.0 (6)	2.1 (7)
Range	0.73-1.5	1.2-3.1
Percent activity in soluble protein fractions*	Activity	
Mean against ATCh (n)	82 (6)	43 (7)
Range	72-93	29-58
Mean against BTCh (n)	90 (5)	66 (7)
Range	77-100	44-77

*At 37°C and pH 7.7.
The activity of the total homogenate was taken as 100%.
n - number of parasite batches.

The hydrolysis of DDVP, E600 (O,O-diethyl-4-nitrophenyl phosphate) and MeE600 (O,O-dimethyl-4-nitro-phenyl phosphate) was studied (37°C; bicarbonate buffer, pH 7.4) in homogenates of Ascaris suum, Fasciola hepatica, M. apri, Neoascaris vitulorum, Paramphistomum microbothrium and Schistosoma mansoni, in plasma and erythrocytes of man, rat, rabbit, hamster and chicken, and in liver homogenates of rat and hamster. All preparations hydrolyzed DDVP (Tables 13 and 14), and, for 10 mM DDVP, the activities ranged from 2 to 60 µmol/h/g wet weight tissue. Mammalian plasma hydrolyzed E600 at about the same rate as DDVP; (Table 14) little or no hydrolysis of E600 (5 mM) and MeE600 (5 mM) was found in vertebrate erythrocytes in chicken plasma and in parasitic helminths. The Michaelis constants for DDVP were all in the millimolar range. The hyrolysis of DDVP in parasitic helminths and in vertebrate plasma was inhibited by $MgCl_2$ and $AgNO_3$, while the hydrolysis in the erythrocytes was unaffected; the non-competitive K_I for rabbit plasma was 5.1 mM and 0.5 µM for $MgCl_2$ and $AgNO_3$, respectively.

TABLE 13. HYDROLYSIS OF DDVP AND PA IN HOMOGENATES OF PARASITIC HELMINTHS*

Species	Hydrolysis/µmol h^{-1} g^{-1} wet weight			
	DDVP 10 mM	n	PA 10 mH	n
A. suum	6.9	12	138	5
F. hepatica	57	2	-	
M. apri	33	1	-	
N. vitulorum	8.6	2	67	1
P. microbothrium	21	6	260	2
S. mansoni	6.6	1	28	1

n = number of parasite batches
*All activities determined manometrically

TABLE 14. HYDROLYSIS OF DDVP AND E600 IN PLASMA AND ERYTHROCYTES*

Species	Hydrolysis/µmol h^{-1} ml^{-1}					
	DDVP 10 mH			E600 5 mH		
	Plasma	Erythrocytes	n	Plasma	Erythrocytes	n
Rabbit	61	5.8	8	93	2.3	6
Rat	12	14	13	18	1.9	9
Man	11	5.6	20	9.1	0.94	14
Hamster	3.7	7.9	1	-	-	-
Chicken	1.9	7.5	17	Not hydrolyzed		7

*All activities determined manometrically.
n = number of parasite batches

Heat inactivation (at 80°C) of the hydrolytic activities in erythrocytes (against DDVP and MeE600) revealed two enzymes, (Table 15). In plasma (at 53°C) there was no evidence of more than one enzyme (rate constants are given in Table 16). Both enzymes in the erythrocytes hydrolyzed the OPs and PA; their activity was fully abolished by proteolytic digestion. The enzymes in erythrocytes were fully soluble (Table 17). In human erythrocytes, a partial separation from hemoglobin was achieved by gel filtration on Sephodex G-100.

The proteins which hydrolyzed DDVP in all studied preparations can be classified under arylesterases (EC 3.1.1.2).

TABLE 15. HEAT INACTIVATION* OF THE HYDROLYTIC ACTIVITIES IN ERYTHROCYTES.

Species	Substrate	Remaining activity/%	n*
Man	DDVP	54	13
Man	MeE600	39	5
Man	PA	Zero	4
Man	ACh	Zero	5
Rat	DDVP	23	2
Rabbit	DDVP	51	2
Chicken	DDVP	29	3

*1 hr at 80°C. All activities determined manometrically.
†n = number of experiments with erythrocytes of different individuals.

TABLE 16. HEAT INACTIVATION OF HYDROLYTIC ACTIVITIES IN PLASMA

Species	k/min^{-1}*	n†
Man	0.026	4
Rat	0.097	2
Rabbit	0.017	2
Chicken	0.002‡	5

*k is the first order rate constant of inactivation obtained with DDVP and E600 as substrates. All activities determined manometrically.
†n is number of experiments with plasma from different individuals.
‡Obtained with DDVP only.

TABLE 17. DISTRIBUTION OF HYDROLYTIC ACTIVITIES BETWEEN STROMA AND HEMOLYSATE IN HUMAN ERYTHROCYTES*

Substrate	Activity µmol h^{-1} ml^{-1} Whole erythrocytes	Percent activity Stroma	Haemolysate	n
DDVP (10 mM)	6.7	Zero	100	5
MeE600	3.2	Zero	100	2
PA (10 mM)	79	49	51	5
ACh (13.8 mM)	140	73	27	5
ATCh (1 mM)	229	61	39	7

*All activities were determined manometrically except towards ATCh which was determined spectrophotometrically.
n = number of experiments.

SUMMARY

Twenty five pyridinium oximes were synthetized and tested *in vitro* for reactivation of phosphorylated and phosphonylated AChE, and *in vivo* against poisoning by OP compounds. Some of the compounds were about equally effective as PAM-2, TMB4 and toxogonin, which are the best known antidotes so far.

The binding sites in AChE for several inhibitors were studied, in order to establish whether the substrate-inhibition-site takes part in the reaction. It was concluded that the substrate-inhibition-site is not involved in the competition between two carboxyl esters, but when the OP compound haloxon is the inhibitor, the reaction occurs close to that site.

Comparative studies of cholinesterases and arylesterases in different species were conducted using DDVP as inhibitor (of ChE) and substrate (of arylesterases). No difference was observed in the kinetics of the reaction in different species, and the activity of the arylesterases (expressed per wet weight) was of the same order in mammalian and in non-vertebrate tissues. The kinetics of the reaction were also used as a tool for determining the number of ChEs in a given tissue. Further, the return of ChE activity *in vivo* after inhibition by DDVP was used to evaluate the rate of synthesis of human plasma and erythrocyte ChEs.

SECTION 3

RESIDUES OF ORGANOPHOSPHORUS PESTICIDES IN HUMAN URINE

Alkali metal salts of the dialkyl esters of phosphoric, thiophosphoric and dithiophosphoric acid are produced in the course of hydrolytic and metabolic degradation of OP pesticides. Therefore, a sensitive and specific determination of amounts of these salts in urine and blood samples is of great value in protecting against the bad effects of absorption of OP pesticides. Several procedures have been described for monitoring the absorption of pesticides by humans and animals based on acidification of a urine sample and conversion of the acidic form of the excreted OP species into trialkyl derivatives suitable for gas chromatographic (GC) analysis (Shafik et al. 1973, Blair and Roderick 1976, Lores and Bradway 1977, Bradway and Shafik 1977). An analogous procedure for monitoring these pollutants in surface waters is based on collection of OP species on Amberlite XAD-4, with simultaneous separation of the greater part of the inorganic phosphates (Daughton et al. 1976).

Until recently, in Yugoslavia, a depression of either plasma or blood ChE activity has served as the only indicator for absorption of OP pesticides (Svetlicic and Wilhelm 1973). A significant depression of ChE activity in persons manipulating pesticides was taken as a signal for job-change to avoid further contact with toxicants until the normal cholinesterase activity was restored. Urinary metabolite analysis was introduced to detect absorption of OP pesticides at a phase preceding a depression of ChE activity, so protective measures could be taken sooner (Drevenkar et al. 1979a).

The high concentrations of OP pesticide residues found in the morning urine samples of workers occupationally exposed to phosalone led us to investigate the rates of excretion of metabolites in the urine of a volunteer experimentally exposed to phosalone during one working day and then again during three subsequent days. On the basis of the results of these tests, we established the period necessary for the complete excretion of residues and the appropriate time for urine sampling. (Drevenkar et al. 1979b).

We compared two methods, measuring blood ChE inhibition and measuring OP pesticide residues in urine of occupationally exposed persons, to establish which provides the more reliable indication of absorbed OP pesticide.

The results of our work are published in five papers (Drevenkar et al. 1976a, c, 1979b, c, Stefanac et al. 1976) seven communications Drevenkar et al. 1976b, 1977, 1979a, 1978, Frobe and Stipcevic 1977, Vasilic et al. 1978, 1979) and five theses (Frobe 1977, Meczner 1979, Stipcevic 1978).

MATERIALS AND METHODS

Standards

O,O-Dimethyl phosphorodithioate potassium salt (DMDTPK) Lot. No. 5057; O,O-diethyl phosphorodithioate potassium salt (DEDTPK) Lot. No. 4224; O,O-diethyl phosphorothionate potassium salt (DETPK) Lot. N o. 6803; O,O-diethyl phosphate (DEP) Lot. No. 7302, purity 98%, were all obtained from the United States Environmental Protection Agency Repository, Research Triangle Park, NC, U.S.A.

Diazomethane ethereal solution (10±5 mg CH_2N_2/ml) was prepared from N-methyl-N-nitroso-p-toluensulphonamide (Merck, Germany) following the usual procedure (Vogel 1956), or, according to the usual distillation procedure, from N-methyl-N-nitroso urea. N-methyl-N-nitroso urea was a gift of the Laboratory for Organic Chemistry and Biochemistry, Faculty of Science, University of Zagreb.

Ion-exchanger Amberlite IR-120, H^+-form, 28-35 mesh, was obtained from Fluka AG, Switzerland.

Instruments

Varian Aerographs, Series 1400 (columns II andd III) and Series 2800 (columns Ia and Ib), equipped with Alkali Flame Ionization Detectors with a rubidium sulphate tip and a Pye Unicam GC 204 GC (column IV) with an Flame Photometric Detector were used.

The GC columns used were: glass, 1.8m x 2 mm i.d., packed with 4% SE-30 + 6% OV-210 on 100-120 mesh Gas Chrom Q. (columns Ia and Ib); stainless steel, Im x 2 mm i.d., packed with 25% Triton X-305 on 80-100 mesh Chromosorb W-AW/DMCS (column II); glass, 1.5 m x 2 mm i.d., packed with 25% Triton X-305 on 80-100 mesh Chromosorb W-AW/DMCS (column III); glass, 2m x 2 mm i.d., packed with 4% SE-30 + 6% OV-210 on 100/120 mesh Gas Chrom Q.

The working conditions are listed in Table 18.

TABLE 18. GC WORKING CONDITIONS

Condition	Columns				
	Ia	Ib	II	III	IV
Column temp., °C	190	115	175	140	110
Injector temp., °C	235	160	230	190	250
Detector temp., °C	255	175	235	235	250
Nitrogen carrier flow, ml/min	35	30	35	30	30
Air flow, ml/min	235	235	235	235	30
Hydrogen flow, ml/min	35	35	35	35	30

Methyl derivatives were identified with a GC-mass spectrometer (MS) system by courtesy of Professor D. Jeremic (Faculty of Science, University of Belgrade). A Varian Aerograph Series 1200 GC, equipped with a Thermal Conductivity Detector (TDC), was coupled with Varian MAT CH 5 MS by means of a separator. Ionization source temperature was 190°C, with electron energy of 70 eV. The column was heated to 80°C and 110°C for the determination of the products obtained by methylation of DMDTPK and DEDTPK respectively, and helium was used as a carrier gas.

Methods

Simulated samples, prepared by adding a definite amount of standard salt to urine of nonexposed persons, were treated by three different procedures.

Procedure I--

A dilution series of the standard aqueous DEDTPK solution (4 µg/ml) was prepared. Aliquots of 0.5 ml were added to 2.0 ml of water or urine containing no DEDTPK. Four 2-ml portions of each urine sample were measured into separate 10-ml centrifuge tubes. A known amount of DEDTPK was added to two of them as internal standard. Successively, 2 g of sodium chloride, 4 ml of diethyl ether and 1 ml of 6 N hydrochloric acid were added. The solution was shaken for 1 min on a Vortex mixer, then centrifuged at 425 G. An aliquot of 2 ml was transferred from the organic layer into a test-tube fitted with a ground-glass stopper. One ml of ethereal diazomethane solution was added and mixed in thoroughly, and the tube was left for 10 min. The solution was evaporated to 1 ml under a stream of nitrogen and made up with water to 5 ml. Then, 4 g of sodium chloride was added, and the mixture was extracted with 2-ml portions of hexane. The combined extracts were evaporated to 1 ml in a stream of nitrogen, and subjected to GC analysis.

Procedure II--

To a 5 ml aliquot of a urine sample, 0.5 ml of standard salt dissolved in bidistilled water (or bidistilled water alone in the case of a blank) was added. Inorganic phosphates were removed by adding 0.1g $Ca(OH)_2$, shaking for 1 min on a Vortex mixer, and centrifuging at 425 g for 3 min. One ml of the supernatant was transferred into a test tube with a ground stopper; and 4 ml of diethylether and 1 ml of 6 N HCl was added. This mixture was shaken again on the Vortex mixer and 2 ml of the ethereal layer was separated, and then alkylated by the addition of 1 ml of diazomethane solution. The sample was left to stand for 10 min, and then evaporated under a stream of nitrogen to 1 ml, which also removed the excess of diazomethane.

Procedure III--

The samples were treated according to the method of Blair and Roderick (1976). For the analysis of urine samples collected from the persons occupationally exposed to quinalphos, 9 ml of the final sample obtained by this procedure was transferred into a test tube with a ground stopper and evaporated under a stream of nitrogen to 1 ml.

Recovery rates for Procedures II and III were determined using solutions prepared in the following way: 3 - 5 mg of standard salt was dissolved in 2 - 3 drops of 6 N HCl and 5 ml of benzene was added. After being mixed in a

Vortex mixer for 1 min, 3 ml of the benzene layer was separated and alkylated with diazomethane. The excess of CH_2N_2 was removed and a dilution series of reference solutions prepared.

Sample solutions for GC-MS characterization of DMDTPK andd DEDTPK methyl derivatives were prepared in the same way.

Standard solutions for quantitative evaluation of malathion (Procedure III) and phosalone (Procedure II) residues were prepared by adding amounts increasing from 0.1 to 0.5 ml of water solutions of DMDTPK (0.230 mg/ml) and of DEDTPK (0.11 mg/ml), respectively, to 5 ml urine samples of non-exposed persons.

Control samples for quantitative evaluation of quinalphos andd phosalone residues (Procedure III) were prepared by adding 0.1 to 0.5 ml of DEDTPK (0.0134 mg/ml), DETPK (0.01123 mg/ml) and DEP (0.0107 mg/ml) to 5 ml of urine of a non-exposed person.

Blood and plasma ChE activities were measured as described by Ellman et al. 1961.

RESULTS AND DISCUSSION

Gas Chromatographic Determination of Alkali Metal O,O-Diethyl Phosphorodithioate Present in Trace Amounts

Pesticide formulations containing phosalone are widely produced in Yugoslavia for agricultural and domestic use so there is a demand for an analytical method for assessing exposure and pollution with respect to this pesticide. To meet this demand, a determination of O,O-diethyl phosphorodithioate alkali metal salts was worked out as being the best indicator of phosalone (Drevenkar et al. 1979c). Metabolic degradation of phosalone causes the urinary excretion of alkali metal salts of the diethyl esters of dithiophosphoric, thiophosphoric and phosphoric acid. The last two of these species might also result from derivatives of thiophosphoric acid, and the last one from derivatives of phosphoric acid. Therefore, the determination of alkali metal salts of diethyl phosphorodithioate (Procedure I) was chosen because these can only come from diethyl esters of dithiophosphoric acid, which include phosalone.

Various diazoalkanes were applied for conversion of the salts into the more volatile trialkyl derivatives. Diazopentane is repeatedly recommended (Shafik et al. 1073, Lores and Bradway 1977) as promoting the GC separation of the amyl derivatives obtained from all OP moieties possibly present. A drawback reported in the literature was that the reagent for the preparation of diazopentane is not easily accessible (Blair andd Roderick 1976). We encountered additional problems related to the purity of prepared diazopentane. The GC obtained with blanks showed an abundance of peaks, one of them having the retention time expected for O,O-diethyl-S-amyl phosphorodithio-ate. All attempts to separate the impurities efficiently enough to remove the interference in the GC analysis were unsuccessful. These draw-backs were eliminated when diazomethane, purified by distillation was used as

alkylating reagent. The clean up procedure on a silica gel column, unavoidable after the alkylation with diazopentane, could be omitted. Interferences in the GC analysis arose only when the diazomethane concentration exceeded 15 mg/ml, but such concentrations are easily avoided by dilution, without affecting the results.

The alkylation product of the DEDTPK with diazomethane was characterized by GC-MS.

The retention times of the methylated O,O-diethyl phosphorodithioate are 75 sec on column Ia and 190 sec on column II, with a detection limit signal-to-noise ratio 4:1 of 40 pg.

Systematic errors arising from incomplete methylation and recovery, as well as from changes caused by variations in working conditions during the GC analyses were corrected by running standards and samples alternately. Peak-height measurement was used for quantitative evaluation.

The calibration graph obtained with a series of aqueous samples covering the range 0.04 - 1 ng of DEDTPK was a straight line (slope b=6.80; standard error of the slope s_b=0.55; variance about the regression s^2= 0.86). The detection limit clearly shows the necessity of including an efficient collection procedure (Daughton et al. 1976) in the pollution control of surface waters. A complete separation of inorganic phosphates is not critical, because of the good differentiation of trimethylphosphate in GCs.

For urine samples in the same concentration range, the calibration graph was non-linear.

The results of a series of analyses performed on urine samples of occupationally exposed persons sre listed in Table A-1 (Appendix). In the urine samples of all 12 persons working in the production of pesticide formulations containing phosalone DEDTPK was found. Only the two highest values were accompanied by a depression of the AChE activity in the blood, thus affirming the usefulness and reliability of our method for early detection of exposure. For definite proof of the relation of concentration of metabolites to depression of AChE activity, a GS/MS system with a capillary column is necessary.

Comparable results have been obtained using the procedure described and the particularly simple one of Blair and Roderick (1976) to analyze exposure monitoring. However, a common drawback has to be pointed out. The procedures based on alkylation with diazomathane are not feasible for simultaneous determination of all OP pesticide residues. The necessity of removing inorganic phosphates seriously complicates the sample-treatment procedure when O,O-dimethyl phosphate has to be determined. This drawback does not apply when the alkylation is done with diazopentane, but then there is the problems of obtaining a sufficiently pure reagent for preparation of the diazopentane.

The Rate of Urinary Excretion of Phosalone Residues in Occupationally Exposed Persons

The absorption of OP pesticides by two groups of occupationally exposed workers was evaluated by determining the residues in urine samples (Drevenker et al. 1979b). One group of persons was exposed to malathion and the other to phosalone. To assess the extent of exposure, urine samples were analyzed for DMDTPK and DEDTPK.

The identity of O,O-dimethyl-S-methyl phosphorodithioate, the only product obtained by methylation of DMDTPK, was confirmed by GC-MS analysis.

Procedure III was chosen to test for malathion because it yielded 73 to 89% recovery of DMDTPK from spiked urine samples compared to only 37 to 57% recovery by Procedure II. For phosalone, Procedure II was preferable, giving slightly better recovery (89 to 107% as opposed to 87 to 99%) of DEDTPK than Procedure III.

Urine samples of workers exposed to malathion were collected at the end of work hours, whereas blood ChE activities of some exposed persons were measured a day later. The results obtained are listed in Table 19. DMDTPK concentrations found in ten out of a total fourteen urine samples were in the range from

TABLE 19. ABSORPTION OF MALATHION AND ChE ACTIVITY

Exposed workers Initials	Conc. ng DMDTPK/ml of urine	ChE activity % of pre-exposure value
P.M.	1072 ± 76	-
L.P.	760 ± 65	-
G.A.	690 ± 62	-
Z.A.	641 ± 85	-
K.D.	641 ± 91	-
S.I.	608 ± 49	65
H.M.	545 ± 149	85
K.B.	449 ± 80	95
F.A.	474 ± 80	-
B.J.	471 ± 24	-

449 to 1072 ng/ml of urine. In remaining urine samples, as well as in urine samples of nine non-exposed persons, the malathion residue was not detected. Detection limit was 20 pg.

As only three workers had their ChE activities measured before exposure, the relevant data are for the most part missing in the Table 19 but the highest concentration of malathion residue in urine is accompanied by the lowest blood ChE activity and vice verse. Two workers (not listed in Table 19) from the exposed

group with no metabolite detected in urine had blood ChE activities 96 and 100% of the pre-exposure values.

To check if urinary metabolite excretion is completed within a 24-h period, urine samples of two workers exposed to phosalone collected before and after work were analyzed. Table 20 shows that the concentrations of the residue of phosalone, DEDTPK, in urine samples collected before work were,

TABLE 20. ABSORPTION OF PHOSALONE IN TWO VOLUNTEERS*

Workers Initials	Conc. ng DEDTPK/ml of urine	
	Beginning of Day	End of Day
O.M. Day 1	314 ± 15	311 ± 71
O.M. Day 2	405 ± 5	317 ± 34
O.M. Day 3	347 ± 4	739 ± 122
B.M. Day 1	1274 ± 42	1084 ± 94
B.M. Day 2	864 ± 10	476 ± 30
B.M. Day 3	913 ± 43	474 ± 63

*All results are the mean vslues of two determinations.

within the range of experimental error, equal to, or higher than, those found in urine samples collected after work. The results indicate that the excretion of phosalone metabolites is still going on at the beginning of the next working day.

To verify this conclusion, the rate of DEDTPK, DETPK and DEP excretion in the urine of a volunteer experimentally exposed to phosalone was investigated (Drevenkar et al. 1979b). The experimentally exposed person, who had no previous professional contact with pesticides, worked in the same locations, and performed exactly the same jobs, as other workers.

The results, showing concentrations of metabolites in urine after an exposure lasting 6 h, are presented in Table 21. Urine sampling was carried out at 2 - 4 hour intervals.

In the case of exposure during three succeeding working days (Table 22), urine samples were taken at the beginning and at the end of the work, and 4 -5 hours after termination of exposure. The 24-h urine was also collected, and blood ChE activity before work was measured.

The concentrations of DEDTPK shown in Tables 21 and 22 are expressed in nanograms per milliliter of urine. The concentrations of DETPK and DEP are expressed in peak heights relative to the peak height of O,O-diethyl phosphorodithioate methyl derivative, which was used as internal standard. Due to this difference in expressing concentrations, only the elution patterns, not the absolute amounts, of excreted compound can be compared.

TABLE 21. EXCRETION OF METABOLITES AND ChE ACTIVITY IN A WORKER EXPOSED TO PHOSALONE FOR SIX HOURS

Hours After Exposure		Amount of metabolite*			ChE Activity in Blood (% of preexposure value
		DEP	DETPK	DEDTPK	
Exposure	0.0	Not detected	Not detected	Not detected	100
	0.2	Not detected	Not detected	Not detected	
	4.1	30	84	202	
	6.6	36	186	565	
	8.0	Not measured	Not measured	Not measured	88
	11	195	1246	1994	
	14	92	250	439	
	16	60	124	224	
	21	50	85	136	
	25	44	70	65	
	26	Not measured	Not measured	Not measured	80
	28	36	39	51	
	32	55	48	72	
	36	51	37	45	
	41	48	38	59	
	46	13	10	9	
	49	Not detected	8	7	
	53	Not detected	10	7	

*$\frac{\text{Peak height of DEMP, DEMTP, or DEMDTP}}{\text{ng DEDTPK}}$

Peak height of DEMDTP/ml of urine

TABLE 22. EXCRETION OF METABOLITES AND ChE ACTIVITY IN A WORKER EXPOSED TO PHOSALONE FOR THREE DAYS

Hours After Exposure		Amount of metabolite[a]			ChE Activity (% of preexposure value)	
		DEP	DETPK	DEDTPK	Blood	Plasma
Exposure	0.0	Not detected	Not detected	Not detected	100	100
	5.5	Not detected	Not detected	Not detected		
	8	Not detected	Not detected	Not detected		
	10	10	16	14		
Exposure	25	19	12	24	78	90
	30	18	62	59		
	35	77	195	300		
Exposure	49	20	14	24	88	85
	53	38	57	87		
	58	98	98	151		
	73	48	19	40		
	77	67	29	55		
	83	41	27	27		

[a] $\frac{\text{Peak height of DEMP, DEMTP, or DEMDTP}}{\text{Peak height of DEMDTP/ml of urine}}$ ng/DEDTPK

The observed kinetic patterns show an obvious similarity in the rate and duration of excretion for all three phosalone metabolites. The amount of residues excreted in urine increases gradually and the highest value is reached 4 - 5 h after exposure is ended. Subsequently, the amount decreases abruptly, but at the start of the following working day it is still measurable, and it shows a systematic increase when exposure is prolonged for several following days.

It is also obvious (see Table 23) that metabolite quantities vary during

TABLE 23. METABOLITES IN URINE OF A WORKER EXPOSED TO PHOSALONE FOR THREE DAYS

Days After Exposure		Amount of metabolite		
		DEP	DETPK	DEDTPK
Exposure	1	13	19	16
	2	60	128	188
	3	82	80	118
	4	27	13	24
	5	4	Not detected	Not detected

*$\frac{\text{Peak height of DEMP, DEMTP or DEMDTP}}{\text{Peak height of DEMDTP/}\frac{\text{ng DEDTPK}}{\text{ml of urine}}}$

the period of excretion. The total amount of all metabolites excreted during one day is certainly a better indicator of exposure than metabolite concentration in a single urine fraction. However, the difficulties involved in collecting a total volume urine sample make such sampling inconvenient in a routine monitoring of exposure among workers engaged in the production and application of OP pesticides. Instead of the total excretion of metabolites, the concentrations of DEDTPK in urine sample taken the same period of time after exposure can be used as a satisfactory index of exposure.

Blood and plasma ChE activities were only slightly reduced during exposure; 10 to 20% depression cannot be regarded as a significant indication of exposure.

Although the rate of pesticide degradation varies from individual to individual, and is probably different for different compounds, similar kinetic patterns can be expected for other OP pesticides. It follows that in a pesticide monitoring program designed for control of occupational exposure, routine analysis of urine samples taken 4 - 5 h after exposure offers the most reliable information about pesticide absorption. However, such a sampling procedure is not convenient for routine control, and analyses of urines taken before and after work have to be performed instead. A systematic increase in the concentrations of pesticide residues in the morning urine demands that more efficient measures of protection be taken or that workers be transfered to other jobs.

Human Blood ChE Activities and Quinalphos Pesticide Residues in Urine of Occupationally Exposed Persons

The absorption of quinalphos in a group of workers from a pesticide formulating plant was studied by determining the metabolic degradation product (DEP and DETPK) of this OP compound in their urine samples. DEDTPK was also detected in most of the samples because 1-2 days before starting to work with quinalphos the same workers were making phosalone.

The level of excreted alkyl phosphates in the urine was correlated with the level of ChE activity in the blood of these individuals. Urine samples were collected at the beginning and at the end of the working hours of one day at intervals of 30 days during three months - May, June and July - and immediately after one month's vacation in September. The blood samples were collected before the beginning of the work in the same working day as urine was taken.

For the determination of DEP, DETPK and DEDTPK the urine samples were treated according to Procedure III. The GC analysis of DEP methylated derivative was performed on column III, and GC analysis of DETPK and DEDTPK methylated derivatives on column IV.

Simultaneously with the eleven urine samples of occupationally exposed workers, eleven urine samples of non-exposed persons were analyzed. No alkyl phosphates were detected in the samples from non-exposed workers.

The results of the three month measurements of the eleven workers exposed to quinalphos are listed in the Appendix Table A-2. Note that workers B.D. and U.B. did not have ChE activities measured.

Tables A-2 shows that the amounts of alkyl phosphates in exposed workers vary widely.

On the first working day after one month vacation small amounts of residues were detected at the end of the work only in the urine samples of workers K.F. (67 ng DETPK/ml) and K.M. (158 ng DEP/ml, 123 ng DETPK/ml and 37 ng DEDTPK/ml). The values of ChE activity of all workers were between 80-100% with the exception of the worker K.M. whose whole blood and plasma ChE activities were 28% and 65% of base values. The highest concentration of residues (in K.M.'s urine sample collected in July) was followed by greatest decrease of ChE activity.

Since the workers alternate every 3 to 5 days between work with OP pesticides and work with some less toxic carbamates, and activity of blood ChE in the exposed persons is affected by both classes of compounds, a comparison between the amounts of DEP, DETPK and DEDTPK and the whole blood and plasma ChE activities can hardly be made. Moreover, the OP compounds and carbamates are often formulated at the same time in the same room. Therefore, in this case, ChE activity cannot be used as a parameter indicating the intensity of exposure to quinalphos or OP pesticides only; because it is caused by absorption of ChE inhibitors in general. On the other hand, the quantity of

excreted residues specifically indicates the absorption of the parent OP pesticide, and is a sensitive and selective method for early detection of harmful exposure to these compounds.

SUMMARY

A GC determination of traces of alkali metal O,O-diethyl phosphorodithioates is described. The salts were converted into a volatile derivative by alkylation with diazomethane. The product was identified by GC-MS. A linear relationship of peak height to amount of salt was obtained in the range 0.04 - 1 ng with aqueous samples, and a calibration curve was constructed for urine samples. The detection limit was 40 pg of salt. The procedure was successfully used for monitoring phosalone absorption by occupationally exposed persons.

The absorption of malathion and phosalone was followed in occupationally exposed workers by determination of residues excreted in the urine. Because of the high concentrations found in the morning urine samples, the rates of excretion of phosalone metabolites in the urine of a volunteer experimentally exposed to phosalone during one, and then again during three subsequent, working days were investigated. The urinary excretion of phosalone metabolites was most intense 4 to 5 h after exposure. At the beginning of the next day, the metabolites were still measurable in the urine. Blood and plasma ChE activities were only slightly reduced during exposure. The analyses of 24 h urine samples, or of urine samples taken 4 to 5 h after exposure , are not convenient for the routine monitoring of occupationally exposed persons because of sampling difficulties. Instead, analyses of samples taken immediately before and after work hours have to be performed. A systematic increase in the concentrations of pesticide residues in the morning urine indicates the need for more efficient protection measures.

A study comparing blood ChE inhibition to OP pesticides residues in urine of occupationally exposed persons was carried out. The quantity of an excreted residue is a reliable indication of the absorption of a given OP compound. Blood ChE inhibition, however, reflects the effect of any absorbed compound which inhibits these enzymes.

SECTION 4

RESIDUES OF CHLORINATED HYDROCARBONS IN HUMAN MILK AND BLOOD

The use of chlorinated hydrocarbons has recently been restricted in Yugoslavia. In 1975 we initiated a survey to establish the degree to which these pesticides have been absorbed by the general population and by occupationally exposed workers. The survey, which includes hexachlorocyclohexane and DDT, should provide a basis for a later study to follow-up the effects of the recent restrictions. More recently, we also started a survey, in collaboration with WHO/UNEP, of DDT found in human milk, and in mothers' and cord blood serum.

We have previously published the results of our work in three papers (Krauthaeker 1980a, b, Reiner 1977a) and two communications (Krauthacker 1977, 1978). For details of the experimental procedure, and for references to the literature, the reader is referred to these publications.

MATERIALS AND METHODS

Blood samples from the general population and from occupationally exposed workers were collected in four different parts of Yugoslavia. Human milk and samples of mothers' and cord blood were collected in one maternity hospital.

The pesticide residues were determined by GC. All compounds were identified by their retention times as compared with known standards.

RESULTS AND DISCUSSION

Residues in Blood Serum of the General Population and in Occupationally Exposed Workers

Levels of chlorinated hydrocarbon pesticide residues in serum samples of the general population of four different parts of Yugoslavia, and in two groups of occupationally exposed workers were determined by GC (see Appendix, Table A-3). Serum samples, 262 from the general population and 78 from exposed workers, were analyzed for p,p^1-DDT, p,p^1-DDE,p,p^1-DDD, Lindane, and α-HCH. All samples contained over 1 µg/l p,p^1-DDE. The concentrations of residues in the general poupiation fall within the range reported for other countries. Few samples from the general poupiation contained Lindane or α-HCH; the means for Lindane were between 3.1 µg/l and 3.8 µg/l and for α-HCH were between 1.3 µg/l and 2.6 µg/l. However, the means for the exposed workers were much higher, 9.2 ng/l for one group and 38 µg/l for the other.

Residues in Human Milk, and in Mothers' and Cord Blood Serum

All samples were collected in a continental town of Croatia from 1977 to 1979.

Two extraction methods for milk were used and compared. Method A was recommended by the Environmental Protection Agency, USA (Thompson 1974) and method B was described by Curley and Kimbrough (1969).

Method A--One ml milk was extracted three times with 2.5 ml acetonitrile. To the combined acetonitrile extracts 25 ml 2% Na_2SO_4 was added, and the mixture was extracted three times with 5 ml hexane portions. Hexane extracts were purified on a florisil column (1 cm diameter) containing 1.6 g florisil topped with 1.6 g Na_2SO_4 anhydrous. The column was prewashed with 20 ml hexane. The organochlorine compounds retained on the column were then eluted with hexane until the collected eluates had a total volume of 25 ml. The eluates were evaporated to dryness in a stream of nitrogen, the compounds redissolved in 1.0 ml hexane and analysed by GC.

Method B--One half ml milk was partitioned with 2 ml methanol and 3 ml 1% K_2CO_3. The mixture was extracted three times with 5 ml hexane portions. Hexane extracts were evaporated to dryness in a stream of nitrogen, the compounds redissolved in 1.0 ml hexane, and determined by GC.

Concentrations of p,p'-DDE, p-p'-DDD, and p,p'-DDT were determined in 34 samples of human milk obtained 3-5 days after delivery (Table 24) and in 37 samples obtained from 1.5 to 55 week after delivery (Appendix, Table A-4). All samples contained p,p'-DDE, but only a few samples contained p,p'-DDD and p,p'-DDT. The mean concentration of p,p'-DDE was 31 µg/l in the samples analyzed in the beginning of lactation, and 53 µg/l (By method A) for the samples taken over a 55 week period . The concentration ranges for the first group were 9 -97 µg/l and for the second 9.4 -167 µg/l, and the difference in the mean values is not significant.

The two methods of extraction of milk samples compare fairly well for p,p' -DDE (Appendix Table A-4). Chromatograms of the samples extracted with method B have fewer signals due to impurities than samples extracted by method A, although the latter includes purification on florisil columns.

The content of DDT derivatives in human milk in Yugoslavia falls within the middle of the range reported for non-European and other European countries (WHO 1979). Comparison of our results with those of three other laboratories in Yugoslavia may be found in the Appendix Table A-5.

For the extraction of residues from human serum the method of Dale et al., (1970) was used. To 1.0 ml serum, 1 ml formic acid was added. The mixture was extracted four times with 3 ml hexane portions by shaking on a Vortex mixer for 1 min, and centrifuging for 5 min to ensure separation of phases. The combined hexane extracts were washed with 1 ml 5% K_2CO_3 and then purified on a florisil column (1 cm diameter) containing 1 g florisil topped with 1 g Na_2SO_4

TABLE 24. CONCENTRATIONS OF CHLORINATED HYDROCARBONS

Compound	SERUM						MILK*	
	Mothers (N† = 35)		Non-Pregnant (N = 24)		Cord (N = 35)		N = 34	
	N‡	Conc§ (µg/l)	n	Conc (µg/l)	n	conc (µ/l)	n	Conc (µg/l)
P,P'-DDE	35	18 (8.9 - 46)	24	20 (3.7 - 75)	35	6.8 (3.6 - 17)	34	31 (9.0 - 97)
p,p'-DDD	2	2.9 (2.8 - 3.2)	3	8.3 (6.7 - 12)		Not Detected	5	9.7 (7.0 - 11)
p,p'-DDT	7	5.5 (3.8 - 8.5)	5	14 (4.7 - 27)	2	4.8 (4.0 - 5.8)	5	11 (4.4 - 20)

*Samples of human milk taken 3-5 days past partum, extracted by method A.
†d = number of samples analyzed.
‡m = number of samples containing the particular compound.
§Concentrations are expressed as geometric means.

anhydrous. The column was prewashed with 20 ml hexane. The organochlorine compounds retained on the column were then eluted with hexane until the collected eluates had a total volumn of 25 ml. The eluates were evaporated to dryness in a stream of nitrogen, the compounds redissolved in 1.0 ml hexane and analyzed by GC.

SUMMARY

The concentrations of DDT and hexachlorocyclohexane residues in blood in the general population of Yugoslavia fall within the range reported for other countries. Workers engaged in the production, formulation and packing of pesticides have a higher incidence, and higher levels, of residues than the general population.

Serum samples from non-pregnant women have the same DDT content as serum samples of mothers' at delivery.

Cord blood serum contains lower concentrations of p,p'-DDE than the mothers' serum; the mean ratio for the individual mother/child pair was 2.7. Concentrations in mothers' milk were 2.1 times higher than in the mothers' serum.

SECTION 5

ASSESSMENT OF BIOCHEMICAL AND CLINICAL EFFECTS OF PESTICIDES IN HUMANS

Since the use of pesticides is unavoidable, it is necessary to protect workers exposed to them from their adverse effects. The aim of this work was to relate clinical symptoms in workers to the degree of exposure to a wide variety of pesticides. We also measured Vitamin A levels and concentrations of DDT in some workers and compared the results to the levels found in persons not exposed to pesticides.

Some of the results of our studies have been published in two papers (Wilhelm and Bradamante 1980, Wilhelm et al. 1979) and two communications (Wilhelm 1979, Wilhelm et al. 1978).

ChE ACTIVITY

To evaluate the absorption of pesticides and prevent overexposure to anti-cholinestrase pesticides, measurements of blood ChE activities of exposed workers were taken regularly.

During the period from 1970 to 1979 a total of 567 industrial workers who worked at some time in any of the three different production lines (dust or wettable powder, emulsion, and household sprays) in one plant were examined. Only 170 of them were continuously employed in the production of pesticides for a number of years (2-14). The other were seasonal workers employed only during periods of intense production.

The workers were exposed to different types of pesticides: OP compounds (phosalone, dimethoate, dichlorvos, monocrotophos, naled, bromophos), carbamate insecticides (carbaryl, dioxacarb, carbofuran), some herbicides, fungicides and other compounds. Plasma and erythroycte ChE activities were determined by a spectrophotometric method (Ellman et al. 1961). The measurements of blood ChE activities were performed in the laboratory, while the sampling (by finger prick) was done either in the laboratory or in the factory where the samples were kept cool until transferred to the laboratory. The results of measurements of ChE activity in the blood and their relation to symptoms and signs of poisoning are given in Table 25. The observed complaints or symptoms were weakness, fatigue, headache, nausea, sweating and vomiting.

The production of OP insecticides increased during the ten-year period of observation. It was expected that with the increased exposure, the number of blood samples with diminished enzyme activities would also increase. However, as Table 25 shows, the greatest number of blood samples with enzyme activity

TABLE 25. BLOOD ChE ACTIVITY AND EVIDENCE OF POISONING

Year	Number of Individuals (Without Complaints/With Complaints)					
	0-24% Inhibition	25-49% Inhibition	50-74% Inhibition	75-100% Inhibition	Total	% Complaints
1970	46/1	10/6	11/13	1/5	68/25	87
1971	59/1	18/14	5/6	0/3	82/24	23
1972	99/0	6/0	0/1	0/0	105/1	.9
1973	47/4	18/1	1/1	0/0	67/6	8
1974	56/0	37/14	9/2	0/1	102/17	14
1975	83/1	71/9	6/8	1/1	161/19	11
1976	89/3	26/4	2/2	0/0	117/9	7
1977	167/3	7/1	0/5	0/3	174/12	6
1978	155/3	9/1	1/12	0/6	165/22	12
1979	183/4	34/6	6/16	0/6	223/32	13

less than 50% of normal were recorded in the first two years, as were the greatest proportion of accompanying symptoms or signs of poisoning.

In the first two years, the workers were exposed to and handled mainly dimethoate and chlorfenvinphos, insecticides which are extremely toxic to mammals. The technological procedures and working condition provided inadequate protection, and, in addition, there was a great deal of overtime work during the peak of the season. After the cases of poisoning were analyzed, working conditions were considerably improved. As a result, although the extent of work remained the same, there were no cases of occupational poisoning in 1972, except for one accidental intoxication at work. In 1973 the situation did not change. Only two exposed workers had blood cholinesterase activities below 50%, that is below the limit for safe occupational exposure.

In the next years there was an increase in the production of insectivides. The number of seasonal workers with no previous experience with insecticides also increased. From the results presented in Table 25 the number of reduced enzyme activities again seems to have become higher. If, however, the accompanying symptoms observed in some workers are expressed in percent they varied between 7 to 14%.

Vitamin A Level

There are two references in the literature which deal with the effect of pesticides on the level of vitamin A in humans. Ember and co-workers (1972) report that the amount of vitamin A is lower in the blood of workers exposed to OP compounds, and Keil and co-workers (1972) claim that the amount of vitamin A is higher in persons exposed to chlorinated hydrocarbons. Although the difference between the control and exposed groups reported by these authors is small, we though it worthwhile to check the data, considering the importance of the role of vitamin A in the human body.

TABLE 26. VITAMIN A IN SERUM OF WORKERS EXPOSED TO PESTICIDES

Description of the group	Duration of exposure (Mean Range)	Date of blood sampling	Vitamin A, IU/ml Mean ± S.D. (n) (Range)	Mean Age (Range)
Workers exposed to different pesticides except chlor.hydr.	2 years (1 - 6)	May 1976	1.54 (17) ± 0.32 (0.75 - 2.0)	25 yrs (20 - 29)
Workers exposed to different pesticides including OP and chlor. hydr.	14 years (4 - 23)	May 1976	1.40 (48) ± 0.48 (0.25 - 2.25)	44 yrs (24 - 63)
	14 years (4 - 23)	June 1977	1.78 (55) ± 0.50 (1.0 - 2.8)	38 yrs (19 - 56)
Control Non-exposed workers	-	April 1977	1.79 (98) ± 0.52 (1.0 - 3.4)	38 yrs
Control Non-exposed workers	-	April 1977	1.76 (33) ± 0.41 (1.0 - 2.25)	37 yrs (20 - 60)
	-	June 1977	1.43 (33) ± 0.31 (1.0 - 2.0)	37 yrs. (20 - 60)

In the course of 1976, the level of vitamin A was determined in the serum of 65 workers exposed to pesticides; in a number of these workers the vitamin A level was again determined in 1977. In the control group, the vitamin A content was also determined twice, in April and in June 1977. The control group was selected from the same factory, but among workers who had no contact with pesticides. All the examines were of the same social status and belonged to the suburban population.

Vitamin A was determined in the serum by the spectrophotometric method with antimonium trichloride (Carr and Price 1926, Kimble 1939). By this method its total amount is determined regardless of whether vitamin A is an ester or an alcohol. In some samples vitamin A was measured immediately after blood sampling (after 3 to 4 hours), while the remaining samples were frozen and stored at -18°C until analysis (a month at the most). From the samples in which vitamin A was determined immediately after sampling, a part of the serum was separated and frozen. When the analysis was repeated (within a month after freezing) there was no difference in the results. During each analysis a calibration curve was made on the same day with retinil acetate as standard solution (U.S.P. - standard; INC-Rochville, N.D. USA).

The results of measurements of vitamin A are given in Table 26. They show no effect of pesticides on the level of vitamin A. All values of the control and the exposed groups are within the same range; the standard levels found in the literature (see Appendix Table A-6) are within this range too.

Besides vitamin A, the total DDT in the serum and ChE activities in blood and plasma were also measured in exposed workers in order to verify that pesticides were absorbed. All workers were exposed to pesticides for a long time, but exposure was not continuous and each of them was without exposure for a certain period of time during the year. ChE activity was therefore monitored before the beginning of work with anticholinesterase pesticides and in the course of the work. In Table 27 ChE activities are expressed as percentage values measured before contact with pesticides or after a longer break in the work with pesticides (1-2 months). At the time of sampling ChE activities were not reduced more than 20% in any of the groups. The mean activities were within the standard deviation of preexposure values. However, several times in the course of work with pesticides the same workers showed considerably reduced ChE activities, proving that they absorbed a certain amount of the compounds they were in contact with. The total DDT content in the exposed groups is given in Table 27. Workers not exposed to chlorinated hydrocarbons (A in Table 27) have a lower level of DDT than those exposed (B). The amount of total DDT in those not exposed to chlorinated hydrocarbons is comparable to the amounts of DDT found in the general population (Krauthacker et al. 1980b).

TABLE 27. ChE ACTIVITY AND DDT IN SERUM OF WORKERS EXPOSED TO PESTICIDES

	A*	B†	
	Mean ± SD (n)	May 1976	June 1977
		Mean ± SD (n)	
Total DDT (ppb)	37 ± 17 (9)	108 ± 78 (41)	-
ChE activity (%)			
- blood	92 ± 12 (17)	88 ± 12 (48)	84 ± 23 (48)
- plasma	92 ± 17 (17)	91 ± 12 (48)	84 ± 24 (48)

*workers exposed to pesticides but not to chlorinated hydrocarbons
†workers exposed to chlorinated hydrocarbons

SUMMARY

The surveillance of workers exposed during the formulation of anticholinesterase insecticides has shown that the use of prescribed protective devices at work and the observation of hygienic and sanitary regulations provides a satisfactory degree of protection and significantly reduces the absorption of anticholinesterase insecticides.

Our study has confirmed that weekly measurement of ChE activity during the period of intense production is a practicable method of determining the degree of absorption. With this method, one can learn in time whether there are workers at risk, and prevent the danger of further absorption.

Without exception, workers showing cholinergic symptoms had their blood ChEs inhibited at least 50%. However, complaints from workers were not always related to ChE inhibition.

Absorption of pesticides does not seem to have an effect on the serum level of vitamin A.

SECTION 6

EFFECT OF PESTICIDES ON THE EYE AND ON VISION

The work described in this section is a continuation of the study of the alleged effect of pesticides on the eye and on vision which was described in our previous EPA report (Reiner 1977b). The literature concerning this topic has recently been extensively reviewed (Plestina and Plukovic-Plestina 1976, 1978).

SUBJECTS AND METHODS

Fifty-seven workers who never suffered from an eye disease were selected for this investigation from those on whom the effects of anticholinesterase pesticides on blood ChE were monitored (see Section 5 for details of their exposure). Group A (47) engaged in the production of pesticides and group B (10) engaged in their application. Age, sex and years of exposure of the 57 are presented in Table 28.

TABLE 28. EXPOSURE TO PESTICIDES FOR TWO GROUPS TESTED FOR EYE DEFECTS

Group N	Description of work	Age (years) $\bar{X}$ ± S.D.	Age (years) Range	Exposure (years) $\bar{X}$ ± S.D.	Exposure (years) Range
A					
Men 25	Production and formulation of pesticides, mostly anticholinesterases	37.2±10.9	19-57	11.3±6.7	1-24
Women		35.6±12.0	21-55	11.4±6.6	1-23
B					
Men 10	Application of pesticides in agriculture and for public health purposes	34.8±10.2	21-52	6.4±6.8	2-20

Only permanent workers, preferably with long exposure to pesticides, were selected. Among them, 18 were engaged in work with pesticides between one and

five years, the remaining 39 for over five years. Only 11 workers were older than 45.

The ophthalmological examinations were performed at the same University Hospital, by the same team of ophthalmologists, and with the same equipment as in previous years, so that the diagnostic criteria were identical throughout the study.

Besides a detailed history of past and present illnesses possibly connected with the workers' eyes and sight, each worker underwent detailed opthalmological examination which included tonometry, ophthalmoscopy, slit-lamp biomicroscopy, keratometry, and visual acuity tests. In most of the workers, peripheral vision was measured on a Goldmann perimeter; in some workers, dark adaptation ability was assessed on a Goldmann-Weekers adaptometer. Whenever an abnormality was found, the worker was examined further as a patient, and all the tests were used to clarify the diagnosis and the etiology of the disease. These patients were then treated with the standard therapeutic procedures.

The details of all ophthalmological examinations were described in the previous report (Reiner 1977b).

RESULTS AND DISCUSSION

Out of the 47 workers from group A, 23 complained of some eye troubles (lacrimation, 13; photophobia, 8; itching and burning of the eyes; 3; other complaints, 2). Among the 10 workers from group B, only three complained of eye problems (photophobia or lacrimation).

The most frequent abnormality of the frontal eye segment was dilated and/or tortous episcleral blood vessels. The other eye problems found as a result of external eye examination are summarized in Table 29.

TABLE 29. EXTERNAL EYE EXAMINATION IN WORKERS EXPOSED TO PESTICIDES

Group	Dilated episcleral blood vessels	Conjunctival injection	Abnormal pupils	Lens opacities
A (N=47)	23	3	3	3
B (N=10)	4	2	0	0

Keratometric measurements revealed no great abnormalities in most of the workers. The results of these measurements are presented in Table 30. In four

TABLE 30. KERATOMETRIC MEASUREMENTS OF WORKERS EXPOSED TO PESTICIDES

Group	Mean±S.D.	Number of eyes with difference in meridians of				
		<0.5	0.6-1.0	1.1-1.5	1.6-2.0	>2.0
A						
Right eye	1.01±0.96	27	38	18	4	7
Left eye	1.07±1.09					
B						
Right eye	1.08±0.57	5	3	5	3	2
Left eye	1.02±0.60					

*Expressed as a difference between Horizonfal and vertical meridian in diopters.

workers from group A, and in one worker from group B, a pronounced astigmatism (*i.e.* the difference between the two vertically placed meridians of over 2 diopters) was found.

In all but one worker from group B, visual acuity, after correction with lenses, was 1.0 or over. Accomodation ability and focussing power, tested with Jager's reading charts, were found normal or appropriate to the age of the worker.

Intraocular pressure was found to be considerably above physiological standards in six workers. However, after thorough examination (repetitive applanation tonometry, gonioscopy, water load tests) an open angle glaucoma was diagnosed in one of the workers. The cause of the increased intraocular pressure in other five workers remained unexplained. The results of intraocular pressure measured by the Schiotz's tonometry or applanation tonometry, are presented in Table 31.

Eyeground abnormalities were found in the patient with glaucoma, as expected, and in several other workers. These abnormalities were mostly mild, degenerative changes of the retina or slight abnormalities of retinal blood vessels, which are as prevalent in the general population as among these workers.

Peripheral visual fields were determined in most of the workers from group A and in four subjects from group B. Peripheral visual fields were found normal in 28 workers from group A and in two workers from group B. Slightly constricted visual fields, mostly for the first and the second isopters, were found in 11 subjects from group A and in two from group B. In five workers from group A, visual fields were considerably constricted in all the four isopters.

TABLE 31. INTRAOCULAR PRESSURE* IN WORKERS EPXOSED TO PESTICIDES

Group	N	Right eye		Left eye	
		Mean	S.D.	Mean	S.D.
A	44	17.0	2.89	16.4	2.83
B	10	13.9	2.47	13.4	2.95

*Measured by Schiotz's tonometry and/or applanation tonometry (in mm Hg).

No feasible explanation for constricted visual fields was found (except for the patient suffering from glaucoma). In the patient with glaucoma, the shape of the constriction of the visual fields was typical for glaucoma. In the others, the visual fields were constricted concentrically for all the four isopters, but mostly for the first and the second.

Dark adaptation ability was measured in 16 subjects from group A. In two workers, adaptation ability was remarkably slower. In the others, no significant differences from standard values was noticed.

SUMMARY AND CONCLUSIONS

Since no group of unexposed workers served as a control in this study, the results of these examinations must be compared with those obtained on control subjects in the previous study. In addition, the evaluation of the results and comparison of the data obtained should be based on the normal physiological values for our population as judged by the experienced opthalmologists.

Considering all the results, from the previous and the present investigations, we did not find that exposure to pesticides had the effect on refraction reported by many Japanese workers in the past. Recently, however there have been several reports from Japan denying any connection between the high incidence of myopia and exposure to pesticides (Sato 1977).

The very high prevalence of dilated and/or tortous episcleral blood vessels is very intriguing since it is considerably higher than normally found in general populations of the same age or among workers not exposed to pesticides (Plukovic-Plestina, unpublished results).

The most consistent abnormality found among the workers exposed to pesticides was constriction of the visual fields of unknown etiology. Any assumption regarding its pathophysiological basis would be speculative.

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APPENDIX A

TABLE A-1. AMOUNTS OF DEDTPK IN URINE OF WORKERS EXPOSED TO OPs

Subject	Amount found, ng/ml 1st sampling	2nd sampling
I.P.	70	254*
	80	266*
S.B.	153	268
	178	272
R.V.	156	
	157	
S.D.	268	
	210	
M.S.	113	
	148	
S.V.	115	
	157	
F.T.	140	
	150	
J.S.	70	
	72	
F.K.	741	115*
	906	147*
I.D.	283	
	389	
M.M.	123	
	128	
S.S.	55	
	46	

*Gas chromatographic analyses were performed on two columns of different polarities (column Ia and II).
The urine samples of exposed persons were taken at the end of a working day and independent analyses performed with two aliquots.

TABLE A-2. METABOLITES IN URINE OF WORKERS EXPOSED TO QUINALPHOS

WORKERS' INITIALS	ng of metabolite/ml of urine						ChE activity (% of preexposure value)	
	DEP		DETPK		DEDTPK			
	A	B	A	B	A	B	Blood	Plasma
May 1979								
A.M	592	308	292	187	285	151	77	58
B.D.	475	275	59	145	69	117		
J.S.	232	86	83	139	ND	43	74	70
K.J.	119	ND	ND	ND	ND	D	85	82
U.B.	249	293	94	153	54	80		
Z.S.	193	193	82	112	83	59	88	88
J.A.	424	359	276	266	137	114	57	35
K.S.	ND	ND	44	57	ND	26	86	100
K.F.	228	134	ND	65	ED	60	83	56
K.M.	680	319	163	181	201	192	88	77
P.S.	731	245	291	152	94	53	100	82
June 1979								
A.M.	99	96	94	73	49	ND	84	58
B.D.	414	214	206	58	82	D		
J.S.	ND	ND	37	ND	ND	ND	73	70
K.J.	ND	ND	ND	ND	ND	ND	68	NM
U.B.	71	ND	74	ND	ND	ND		
Z.S.	ND	67	45	63	37	38	93	83
J.A.	708	704	261	391	86	74	80	47
K.S.	ND	ND	36	68	ND	41	46	76
K.F.	206	73	81	D	42	D	34	42
K.M.	NM	NM	NM	NM	NM	NM	NM	NM
P.S.	NM	NM	NM	NM	NM	NM	NM	NM
July 1979								
A.M.	1896	656	349	381	61	48	79	61
B.D.	564	749	208	657	ND	79		
J.S.	247	106	83	103	54	43	80	73
K.J.	113	ND	68	ND	ND	ND	68	65
U.B.	147	325	120	352	ND	ND		
Z.S.	213	ND	100	128	ND	ND	86	87
J.A.	NM	NM	NM	NM	NM	NM	NM	NM
K.S.	357	168	197	181	D	ND	72	76
K.F.	477	238	203	139	57	D	59	48
K.M.	1807	1245	759	1752	50	78	68	45
P.S.	422	828	245	616	D	86	NM	NM

D = detected
ND = not detected
NM = not measured

TABLE A-3. SERUM CONTENT OF ORGANOCHLORINE COMPOUNDS IN YOGOSLAVIA

Place year	N* (m,f)	Mean age Range		Concentration in µg/l (n)† α-HCH	Lindane	p,p'-DDE	p,p'-DDD	p,p'-DDT
General Population								
Continental north 1975	147 (65,82)	42 8-92	Mean Range	1.8 (57) 0.1 15	3.1 (23) 0.5-15	30 (147) 8.4-118	9.4 (7) 3.0-23	19 (20) 2.2-81
Continental north 1976-1978	11 (6,5)	34 21-47	Mean Range	- -	- -	27 (11) 5.1-70	4.3 (5) 1.3-8.6	4.0 (4) 0.4-10
Continental central 1979	41 (14,27)	35 6-79	Mean Range	n.d. -	n.d. -	6.9 (41) 1.2-31	1.1 (34) 0.5-2.1	2.2 (32) 1.1-5.3
Continental south 1976	19 (19,-)	37 20-69	Mean Range	2.6 (4) 1.4-8.3	3.8 (2) 2.1-6.7	29 (19) 9.2-118	5.2 (10) 3.4-17	6.1 (8) 3.7-17
Island 1977	44 (25,19)	46 17-78	Mean Range	1.3 (8) 0.7-2.5	n.d. -	18 (44) 4.8-83	6.4 (20) 2.5-23	7.2 (17) 3.1-39
Exposed Workers								
Factory year								
Continental north 1976	50 (31,19)	41 20-62	Mean Range	9.2 (7) 3.6-29	10.4 (29) 3.6-30	53 (50) 7.1-213	11 (35) 3.1-318	16 (32) 3.7-96
Continental south 1976	28 (25,3)	35 21-52	Mean Range	38 (28) 9.2-157	26 (28) 4.3-98	23 (28) 4.1-95	6.2 (8) 3.6-11	11 (7) 4.7-29

*N = number of samples analyzed
†n = number of samples containing the particular compound
‡(m,f) = male, female

TABLE A-4. CONCENTRATIONS OF P,P'-DDE IN HUMAN MILK AT LACTATION PERIODS UP TO 55 WEEKS.

Initials	Time After Delivery (weeks)	p,p'-DDE (µg/l) Extraction method: A	B
M.B.	1.5	86	130
M.M.	3	9.4	13
M.A.	3	113	125
P.Lj.	3	102	140
H.M.	3	58	104
S.M.	4	84	141
P.M.	5	51	64
B.V.	6	75	97
P.K	6	69	77
S.D.	7	34	64
S.Z.	7	135	163
B.Bo.	8	19	26
L.B.	9	56	69
N.K.	9	109	75
M.D.	11	120	139
C.V.	12	87	48
B.Bl.	15	67	98
V.M.	16	53	71
M.O.	32	89	119
S.K.	55	73	79
H.Lj.	7	80	121
	20	51	57
	24	63	57
S.A.	32	20	30
	45	9.8	11
P.S.	3	167	77
	7	74	83
	11	44	57
	15	77	108
M.R.	16	12	13
	23	43	56
	31	45	28
	39	49	42
J.D.	3	56	56
	10	39	49
	18	16	15
	27	37	39

Mean age of 25 mothers: 30 yrs (range 24-42). Mean concentrations (expressed as geometric means of 37 samples: method A 52.6 µg/l (range 9.4-167), and method B 60.6 µg/l (range 11-163).

TABLE A-5. CONCENTRATIONS OF P,P-DDE AND P,P'-DDT IN HUMAN MILK IN YUGOSLAVIA.

Republic	Year of Sampling	Number of Mothers	Concentration (µg/l)				
			p,p'-DDE	Range	p,p'-DDT	Range	
Slovenia	1973	14	3000*	1700-6950*	850*	280-2760*	
	1973	14	60†	34-139†	17†	5-55†	
Serbia	1968	Not Stated	102	Not Stated	94	Not Stated	
	1971	3	411	104-720	96	40-216	
	1971	1; 2‡	126; 559‡	104-720	48; 120‡	40-216	
	1974	5; 5‡	192; 142‡	Not Stated	48; 36‡	Not Stated	
Croatia	1976	27	1537*	28-6900*	256*	0-590*	
	1976	27	31†	0.6-138†	5.1†	0-12†	
	1977	34	31	9-97	11	4.4-20	This Report
	1979/79	25	53	9.4-167	10	2.1-22	This Report

*Concentration expressed on fat basis.
†Recalculated from (a) based on 2% fat in milk.
‡The first number refers to mothers of a single child, and the second to mothers of twins.

TABLE A-6. VITAMIN A LEVELS IN HUMAN SERUM

Authors	Vitamin-A IU/ml serum	Number of Individuals	Population
Kirk (1948)	0.5-0.8	202	Non-exposed
Neeld et al. (1963)	1.2	10	Non-exposed
Drujan (et al. (1968)	1.5	50	Non-exposed students
Mandel (1971)	1.3	-	Non-exposed
Ember (1971)	1.2	23	Workers 40 days after exposure
	0.8	23	Exposed to OP compounds
Kell et al. (1972)	1.4	21	Non-exposed workers
	2.3	21	Exposed to chlorinated hyrocarbons
Sauberlich et al. (1973)	1.5-2.5	-	Non-exposed

APPENDIX B

SUMMARIES OF THESES

Z. Frobe: Preparation of Silyl Derivatives for the Gas Chromatographic Analysis of Organophosphorus Pesticide Residues, B.Sc. Thesis, University (1977).

OP pesticides decompose easily, producing salts of corresponding organophosphoric acids. While the original compounds can be directly determined by GC analysis, their residues, organophosphates, have to be first transformed to some more volatile form. The possibility of quantitative determination of OP pesticides residues by GC of silyl derivatives was investigated.

The derivation of O,O-dimethylphosphate (DMP) and O,O-diethylphosphate (DEP) was carried out in acetonitrile with hexamethyldisilazane as silylating reagent. The product obtained was dissolved in carbon tetrachloride and n-hexadecane was added as internal standard for GC. The silyl derivatives were identified with MS interfaced directly with the GC.

Using the ratio of internal standard to silyl derivative peak height as quantitative parameter, linear dependence of detector response on the concentration change of both DMP and DEP were obtained for the tested range 1-6 mg/ml.

The sensitivity of detection with flame ionization detector was 70 µg/ml. The developed procedure is not sensitive enough for most residue analysis problems in biological samples. However, it is suitable for the monitoring of polluted or surface waters with an accumulation procedure from bigger sample volumes included in the sample treatment.

J. Meczner: Simultaneous Gas Chromatographic Determination of Alkali Metal Salts of O,O-Diethyl-, O,O-Dimethyldithio-, and O,O-Diethyldithio-phosphoric Acid, B.Sc. Thesis, University of Zagreb (1978).

Monitoring procedures of persons occupationally exposed to OP pesticides have been improved by a simultaneous GC determination of alkali metal salts of O,O-diethyl-, O,O-dimethyldithio-, and O,O-diethyldithio-phosphoric acid.

In the following concentration ranges of the originally present salts, a linear relationship has been found; 63-1895 ng/mg urine (b=0.0158, a=0.76) for O,O-diethyl-phosphoric acid K-salt, 100-1555 ng/ml urine (b=0.053, a=4.61) for O,O-dimethyldithio-phosph. ac. K-salt, 47-1405 ng/ml urine (b=0.0738,

a= 3.25) for 0,0-diethyldithio-phosph. ac. K-salt. The inorganic phosphates present in urine samples interfere with the determination of the salts of 0,0-dimethyl-phosphoric acid, because the methylation products of the acids obtained by acidification of the salts are gas chromatographically determined species.

R. Plestina: Toxic effects of metrifonate in mammals, Veniam legandi Thesis, University of Zagreb (1976).

Metrifonate, an antiparasitic drug, is an OP compound known also as an insecticide under the name dipterex. In this work, the author presented the results of his investigations in humans and in experimental animals. The effects of metrifonate was studied in children treated against S. haematobium infection and in adults who had the same parasite but also had various types of hemoglobinopathies. Besides very pronounced inhibition of blood ChE no untoward effects of metrifonate were observed in the treated patients.

The experiments on rats revealed that after an intravenous injection of metrifonate, cholinergic symptoms appeared with some delay. This suggest that metrifonate itself is devoid of direct cholinergic action and that a conversion of metrifonate to a biologically active compound takes place in the animals organism. This observation was supported by the results of experiments with intestinal sacs in which it was shown that the inhibitory power of metrifonate increases with an increase of pH of the media. In rats treated with metrifonate, the pattern of plasma and brain ChE inhibition was similar to that in rats treated with sublethal doses of dichlorvos. In rats fed on a diet containing 500 ppm of abate, toxicity of metrifonate remained unchanged, indicating that the potentiation of toxicity for the two OP compounds did not exist.

Z. Radic: Mechanism of Inhibition of Acetylcholinesterase by Some Oximes, B. Sc. Thesis, Univeristy of Zagreb (1979).

The activity of purified bovine erythrocyte AChE (EC 3.1.1.7.) was measured with acetylthiocholine as substrate (0,10, 0,40, 1,0, 5,0 and 10,0 mM). The maximum activity was at 1.0 mM acetylthiocholine. The evaluated Michaelis constant (K_m) and substrate inhibition constant (K_{ss}) were 0.036 mM and 11.13 mM respectively, and the maximum rate of substrate hydrolysis (V) was 1.45 μmol min^{-1} mg^{-1}.

The inhibition of AChE by 2-PAM (0.10, 0.25, 1.0, and 2.0 mM) and toxogonin (1.0, 2.0, 4.0, 8.0, and 12.0 mM) was studied with acetylthiocholine as substrate. The concentrations of 2-PAM were 0.10, 0.25, 1.0 and 2.0 mM, and of toxogonin 1.0, 2.0, 4.0, 8.0 and 12.0 mM; the substrate concentrations were as stated above.. The inhibition of AChE by both inhibitors was slightly competitive with respect to acetylthiocholine. The Hunter and Downs plots for the degree of inhibition as a function of substrate concentration were slightly curved. The hypothesis of Aldrige and Reiner was applied to interpret this result. According to this hypothesis, the enzyme has two binding sites, one is the active site and the other an allosteric site.

Both sites are assumed to bind both the substrate and the inhibitor. Binding of the inhibitor to either site causes enzyme inhibition. The inhibitor constants were evaluated in the following way. Extrapolation of the Hunter and Downs plots to zero substrate concentration gave the dissociation constants for the enzyme-inhibitor complex at the active site (K_3), and these were 0.178 mM for 2-PAM and 4.39 mM for toxogonin. The dissociation constants for the enzyme-inhibitor complex at the allosteric site (K_I) were calculated from the theoretical equation of Aldridge and Reiner for eleven pairs of substrate and inhibitor concentrations. The mean value for 2-PAM was 0.37 mM and for toxogenin 7.9 mM. The relative standard deviation of each constant was about 5%. The narrow range within which the K_I values varied, and is considered evidence that the theoretical equation describes well the inhibition of AChE by the two oximes.

Two techniques were used to measure the enzyme activities, the conventional technique and the stopped flow technique. For the latter technique, the enzyme concentration during assay was 100 times higher than in the conventional technique. The activities of the uninhibited enzyme (expressed per unit weight) were the same irrespective of the technique used. The degree of enzyme inhibition however, was smaller when measured by the stopped flow technique. This was attributed to non-specific binding of the inhibitors onto the enzyme preparation. The amount of absorbed inhibitor was calculated to be 37% for 2-PAM and 45% for toxogonin.

2-PAM and toxogonin catalyze the non-enzymic hydrolysis of acetylthiocholine. These reactions are bimolecular second-order reactions, and the following rate constants (k) were evaluated: 14.1 min^{-1} $mol^{-1}dm^3$ for 2-PAM and 3.9 min^{-1} $mol^{-1}dm^3$ for toxogonin.

The spontaneous hydrolysis of acetylthiocholine was measured over a wide range of time intervals. The reaction was first order and the corresponding rate constant was evaluated as 5.7×10^{-5} min^{-1}.

All experiments were done in 0.1 M phosphate buffer pH 7.4 at 25°C.

M. Stipcevic: Determination of Organophosphorus Pesticide Residues by Gas Chromatography, M.Sc. Thesis, University of Zagreb (1978).

The quantity of residues excreted with human urine is a useful parameter that indicates the extent of exposure to the parent OP pesticides. The residues are salts of dimethyl or diethyl phosphate, phosphorothioate or phosphorodithioate. For GC analysis they have to be converted into volatile derivatives. The treatment with diazoalkanes and conversion into trialkyl phosphate is widely applied for this purpose.

When diazomethane or diazoethane are used for alkylation the interference of inorganic phosphate present in urine samples might be a serious problem. It can be eliminated by the alkylation with diazopentane, which yields derivatives more easily resolved by GC.

We applied the latter procedure first for the monitoring of persons occupationally exposed to phosalone by determining its residue, O,O-diethyl phosphorodithioate salt. In addition to the difficulty caused by inaccessibility of the reagent for diazopentane preparation, the impurities present in its ethereal extract could not be removed efficiently enough and they spoiled the gas chromatograms. For this reason we chose the alkylation with distilled ethereal diazomethane. The presence of inorganic phosphate did not represent a problem in our case.

In all urine samples of occupationally exposed persons, O,O-diethyl-S-methyl phosphorodithioate was found, and the highest values were accompanied by the lowest values of AChE activity in blood. Analyses of 22 urine samples of unexposed persons did not show the present of O,O-diethyl-S-methyl phosphorodithioate.

Z. Vasilic: Organophosphorus Pesticides in Surface Waters, M.Sc. Thesis, University of Zagreb (1979).

The degradation rate of OP pesticides has to be considered for proper evaluation of the results obtained by analytical methods for the determination of these compounds in surface waters.

The experiments performed with model systems of surface waters with various levels of pollution from various sources, have shown that the degradation rate depends not only on the chemical factors but also on the bacteriological profile of the water supply.

The determination of the total amount of OP pesticides is proposed as an adequate parameter for measurement of pollution of surface waters. This approach requires the determination of diverse compounds of this class taken together and of their degradation products. The analytical method proposeed for pollution control includes 1) hydrolysis of all OP pesticides and of their phosphorus-containing degradation products to phosphoric acid, 2) alkylation with diazomethane and 3) GC determination of trimethyl phosphate. The most critical step is the accumulation of the species to be analyzed along with a complete elimination of inorganic phosphates prior to hydrolysis. The chromatography on an Amberlite XAD-4 column is shown to be promising for this purpose.

V. Vragovic: Quantitative Determination of Some Larvicides by Thin Layer Chromatography, B. Sc. Thesis, University of Zagreb (1976).

The spot detection based on the AChE inhibition with indophenyl acetate as substrate has been successfully applied for the determination of malathion and parathion on thin-layer chromatography. A linear relationship of the spot area, measured densitometrically, to the applied quantity of the pesticide was been obtained in the range 0.9 - 3.5 ng for parathion and 0.2 - 1.0 µg for malathion. Enzyme inhibition could very well be used, for qualitative detection of abate on a fluorescent layer even if only 3 ng was present. For the quantitative determination of abate extracted with chloroform from surface water samples, the measurement of the quenching of the fluorescence of the

spots on a fluorescent layer was shown to be satisfactory. In this way the linearity range for 0.9 to 5 µg of abate was obtained.