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Alkyl Phosphate Metabolite Levels in the Urine of Field Workers Giving Blood for Cholinesterase Test in California

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During the Summer of 1975, a blood cholinesterase (ChE) monitoring study was conducted at farm worker health clinics in California's Central Valley. Blood samples were collected at the clinic during the months of January, May, July and September and tested for ChE activity (KNAAK et al. 1978). During the grape harvest season, in September, 154 urine specimens (110 from males and 44 from females) were collected from field workers in addition to blood samples.

This study reports the level of (1) alkyl phosphates in the urine, using a modification of the test procedure published by SHAFIK et al. (1973) and (2) the level of ChE activity in the blood of these individuals.

METHODS

Apparatus and Glassware. A Varian Aerograph 2100 gas chromatograph equipped with a flame photometric detector operating in the phosphorus mode was used. A 1.8 m x 6 mm o.d. glass column packed with 4% OV-101 on 100-120 mesh Chromosorb W was used. The column was conditioned and treated with Carbowax 20 M according to IVES and GUIFFRIDA (1970) to eliminate tailing of the alkyl phosphate derivatives. The operating conditions of the gas chromatograph were: column, 150° C; inlet, 220° C; detector, 210° C; nitrogen (carrier gas) flow rate, 30 mL per min.

Reagents

Formic acid, 1% formic acid in benzene. Extraction solvent, 1:1 (V/V) acetonitrile and diethyl ether. Anhydrous sodium sulfate, washed in benzene, stored in oven at 120° C until used in the analysis. MN-silica gel, (35-100 mesh) was used without activation (Machacrey, Nagel and Company, Brinkmann Inst., Inc.). Diazopentane reagent, as indicated by SHAFIK et al. (1973). Elution 1, 20 mL, 30% CH_2Cl_2 in hexane. Elution 2, 20 mL, 5% acetone in CH_2Cl_2. Preparation of Amyl derivatives, as indicated by SHAFIK et al. (1973).

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Preparation of Standard Solutions. Solutions containing known concentrations of the Amyl derivatives were prepared by diluting out the derivatized standards with an appropriate amount of hexane. The derivatized alkyl phosphates were quantitated based on peak height. Quantition was based on DMAP (3 ng/mL), DEAP (10 ng/mL), DMATP (3 ng/mL), DEATP (3 ng/mL), DMADTP (5 ng/mL) and DEADTP (5 ng/mL).

Analysis of Urinary Metabolites. A 2.0 mL sample of urine was transferred to a 15 mL glass stoppered centrifuge tube to which was added 2.0 g of NaCl, 4.0 mL of 1:1 (V/V) acetonitrile and diethyl ether. The test tube was immediately shaken for 1.0 min and 2.0 mL of the solvent was transferred to another 15 mL centrifuge tube. Diazopentane Amyl reagent was added until a persistent orange color was produced (2 to 3 mL). The reaction mixture was then allowed to sit for one h and then heated at 50°C for 15 min. Amyl derivatives were concentrated to about 0.5 mL under a small stream of nitrogen. The derivatives were then shaken vigorously for 2 min with a mixture of 5.0 mL of water, 5.0 g of NaCl and 5.0 mL of hexane. The tube was centrifuged and the top hexane layer transferred to a clean test tube.

The mixture was again extracted using 5.0 mL of hexane. The combined hexane extracts were evaporated to 2.5 mL and placed on top of a 2.5 x 0.5 cm column of 35-70 mesh MN-silica gel prepared by adding 1 g of Na_2SO_4 to the top of the column and washing with 10 mL of hexane. The Amyl derivatives of DMTP, DETP, DMDTP and DEDTP were eluted off the column using 20 mL of 30% CH_2Cl_2 in hexane (Fraction I) while the derivatives of DMP, DEP, DEPTh and DMPTh were eluted with 20 mL of 5% acetone in CH_2Cl_2 (Fraction II). The individual fractions (I and II) were concentrated to 2.0 mL for gas chromatography. Aliquots of 5 to 10 uL were injected in the chromatograph.

ChE Determinations. The blood ChE activity was determined using the method of KNAAK et al. (1978) developed for the Technicon AutoAnalyzer II. The method colorimetrically determines the ChE activity of whole blood (intact red cells and plasma) and plasma. The ChE activity of the red cell was obtained by subtracting the activity of the plasma from whole blood. The sample's hematocrit was used in the calculation as indicated.

$$\text{RBC} = \frac{\text{Whole Blood Activity} - \left(\frac{[1-\text{Hematocrit}]}{100}\,[\text{Plasma Activity}]\right)}{\frac{\text{Hematocrit}}{100}}$$

Errors involved in pipetting packed red cell volumes were eliminated by this procedure. The AutoAnalyzer II, set up for ChE analysis, was a dual channel analyzer equipped with a

dialyzer in each channel. The dialyzer in the analyzer channel allowed small molecules such as thiocholine, the hydrolysis product of acetylthiocholine, to be analyzed separately from interferring high molecular weight proteins and intact red cells. In this system, the color reagent (DTNB) is mixed with thiocholine after dialysis. The activity is reported in umoles of -SH released per min per mL of sample.

A plasma standard from Sigma Chemical Co. was used as the enzyme standard, while glutathione was used as a standard source of -SH for instrument standardization.

RESULTS AND DISCUSSION

The published method of SHAFIK et al. (1973) was modified to include a heating procedure (15 min at 50° C) in the formation of the Amyl derivatives and a change in the silica gel column procedure. We used 20 mL of 30% CH_2Cl_2 as Fraction I and 20 mL of 95% CH_2Cl_2 as Fraction II. In this procedure no fractions were discarded. The silica gel used in the procedure eliminated the interfering materials previously encountered by SHAFIK et al. (1973).

Figure 1 (B, C, D) gives the gas chromatograms for the Amyl derivatives of the six standard dialkyl phosphates. The dimethyl and diethyl dithiophosphates chromatographed as indicated in Figure 1B, while the dimethyl and diethyl thionophosphates and their isomers, the dimethyl and diethyl thiolophosphates, chromatographed as indicated in Figure 1C. In the analytical procedure the thionophosphates are first separated from the thiolophosphates on the silica gel column prior to gas chromatography. The thionophosphates elute in Fraction I with the dithiophosphates. Dimethyl and diethylphosphate chromatograph as indicated in Figure 1D.

Figure 1(A) is a typical chromatogram of Fraction I containing the thiono and dithiophosphates. The thiolophosphates, if present in Fraction II, chromatograph just prior to dimethyl phosphate. Figure 2 is a chromatogram of a urine sample (Fraction II) fortified with dimethyl and diethylphosphate. The chromatogram shows the presence of triamylphosphate (TAP) and an unknown compound just prior to TAP.

Table 1 gives the recoveries of the various alkyl phosphates from urines fortified at the 0.1 ppm level. Fraction I off the silica gel column contains DMTP, DETP, DMDTP and DEDTP. Recoveries of these compounds were consistantly high. Fraction II contained DMP and DEP. The DMP and DEP were not recovered to the same extend as were the compounds in Fraction I. The thiolates were not found in Fraction II under these conditions.

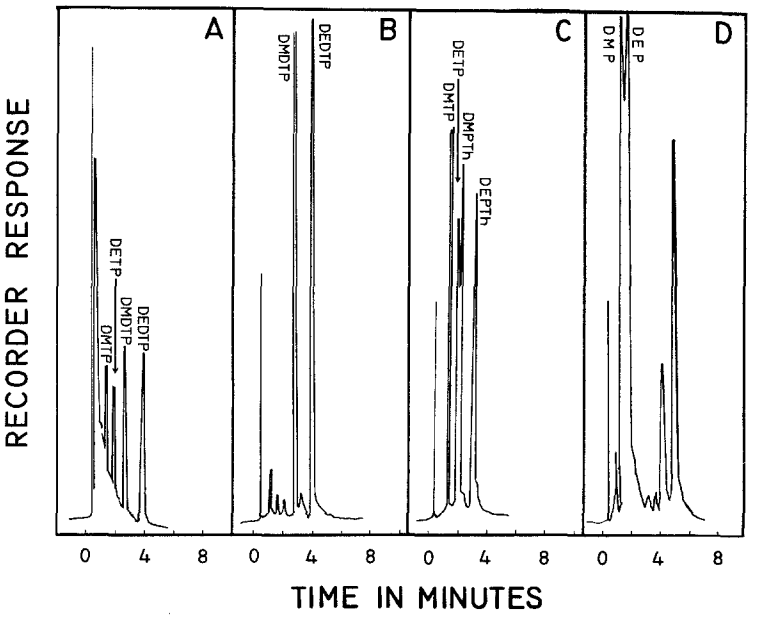

Figure 1

Gas chromatograms of the alkyl phosphates. (A) Urine sample (Fraction I) fortified with Amyl derivatives of DMTP, DETP, DMDTP and DEDTP (1.0 ng each). (B) Standards of DMDTP and DEDTP (5.0 ng each) as Amyl derivatives. (C) Standards of DMTP and DETP (3.0 ng each) as Amyl derivatives. (D) Standards of DMP (3.0 ng) and DEP (10 ng) as Amyl derivatives.

TABLE 1

Dialkyl Phosphates Used in This Study and Their Recoveries From Human Urine Fortified at the 0.1 ppm Level

Derivatized Alkyl Phosphates	Average % Recovery (4 Determinations)	Fraction Off Silica Gel
DMTP $(MeO)_2P(S)OH$	97.1	I
DETP $(EtO)_2P(S)OH$	98.0	I
DMDTP $(MeO)_2P(S)SH$	98.6	I
DEDTP $(EtO)_2P(S)SH$	97.5	I
DMPTh $(MeO)_2P(O)SH$	—	II
DEPTh $(EtO)_2P(O)SH$	—	II
DMP $(MeO)_2POOH$	90.0	II
DEP $(EtO)_2POOH$	89.3	II

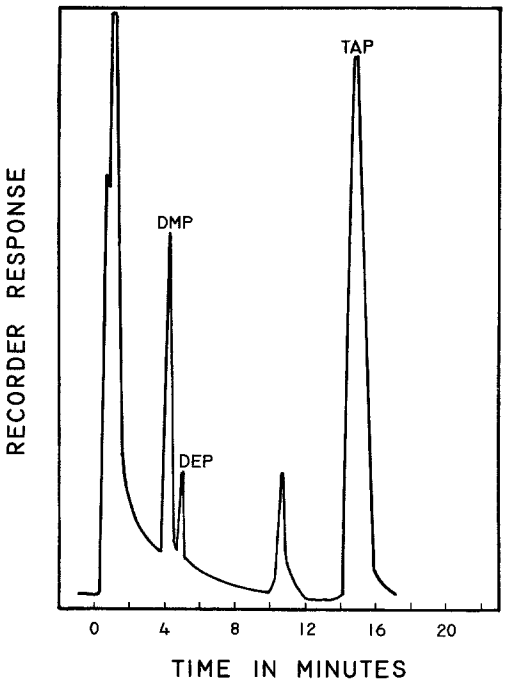

Figure 2

Chromatogram of control urine (Fraction II) fortified with DMP and DEP (5.0 ng each) as their Amyl derivatives.

Of the 154 urines analyzed for alkyl phosphates (110 from males and 44 from females), 25 urines (24 from males and 1 from females) were found to contain trace quantities of DMP, DEP and DMDTP. Diethyl phosphate (DEP) ($\bar{x}$ = 0.069 ppm) was found in 22 of urines from the male field workers, while DMP ($\bar{x}$ = .039 ppm) was found in 2 urines and DMDTP (0.086 ppm) in one urine. A trace (less than .02 ppm) of DEP was found in the urine of the one female field worker.

In Table 2 the results of the blood ChE analysis are given for the 24 males having small quantities of alkyl phosphates in their urines. The plasma and red cell activity of these men were not significantly different from the activity of nonfield working males according to the one-way analysis of variance. For comparison purposes, the frequency distribution statistics for workers and nonworkers are given in Table 2. The range of values (Plasma and RBC) for the field workers was smaller, but within the range of the control group.

TABLE 2

Statistical Analysis of Cholinesterase Values
From Male Field Workers and Nonfield Workers

ANALYSIS OF VARIANCE

Statistic	Plasma		Red Blood Cells	
	Field Workers	Nonfield Workers	Field Workers	Nonfield Workers
Sample Size	24	95	24	95
Mean, $\bar{x}$	9.6	9.4	28.8	27.5
Std Deviation, s	1.41	1.62	4.3	4.1
F Ratio	0.25*		1.95*	

FREQUENCY DISTRIBUTION STATISTICS

Statistic	Plasma		Red Blood Cells	
	Field Workers	Nonfield Workers	Field Workers	Nonfield Workers
Median	9.6	9.4	29.0	27.5
Kurtosis, g_2	-0.67	0.61	-0.39	-0.57
Skewness, g_1	-0.09	-0.18	-0.19	-0.03
Minimum	6.8	3.8	21.0	18.2
Maximum	12.2	13.1	36.8	35.4

*Not significantly different at 5% level. ChE activity expressed in umoles of -SH released/min/mL of sample.

The urine analysis indicates that small amounts of organophosphates were absorbed by the field workers but were not sufficient to cause a decrease in blood cholinesterase values. The values for unexposed individuals varied between 0.02 to 0.04 ppm, while slight exposure resulted in urine levels between 0.04 to 0.1 ppm. Exposure was related to levels of 0.1 ppm and above.

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