A Comparison of Gas Chromatographic and Anti-Cholinesterase Methods for Measuring Parathion Metabolism *In Vitro*

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Parathion, (0,0-diethyl 0-p-nitrophenyl phosphorothionate), a widely used insecticide, is transformed to its oxygen analogue, paraoxon (0,0-diethyl 0-p-nitrophenyl phosphate), a potent inhibitor of acetyl cholinesterase (DIGGLE and GAGE, 1951; GAGE, 1953), by hepatic microsomal enzymes (MURPHY and DUBOIS, 1958; DAVISON, 1955; NEAL, 1967). Hepatic microsomal enzymes also catalyze the oxidative cleavage of parathion to thiophosphoric acid and p-nitrophenol (NEAL, 1967). Because of its high sensitivity and lack of necessity for extensive extraction and cleanup procedures, an anti-cholinesterase assay has been used in this and other laboratories for measuring the enzymatic conversion of parathion (and related phosphorothionates) to its oxygen analogue (MURPHY, 1966; HITCHCOCK and MURPHY, 1971; JOHNSEN and DAHM, 1966; VUKOVICH et al., 1971). Gas chromatography has also been used to measure both parathion (NAKATSAGAWA et al., 1968; RAO and MCKINLEY, 1969) and paraoxon (HOLLINGWORTH, 1969). The purpose of this study was to adapt a gas chromatographic procedure suitable for routine determinations of small amounts of paraoxon produced in tissue incubations in the presence of a large excess of parathion as substrate, and to compare its utility with that of the anti-cholinesterase method of assaying for paraoxon. It was desired that the method be suitable for determining the rate of total metabolism of parathion as well as the production of the active metabolite, paraoxon. A gas chromatographic procedure was developed which used OV-101 (a methyl silicone resin) as a liquid phase and a flame photometric detector for quantitative analysis (BOWMAN and BEROZA, 1970). The procedure is similar to one reported by Jaglan et al., (1969, 1970) for determining the activation of methyl parathion.

The interchangeability of the gas chromatographic and anti-cholinesterase assays for determination of paraoxon was tested by performing analyses by both methods on the same samples. The samples were incubation mixtures of parathion with liver homogenates prepared from mice subjected to different treatments. In order to test the applicability of the methods for investigations of factors that influence parathion metabolism, livers were obtained from control mice or from mice that had been pretreated with piperonyl butoxide. Piperonyl butoxide has been reported to have a biphasic effect on liver microsomal enzyme activity, causing inhibition at 1 hr after intraperitoneal administration, followed by an increase in activity after 24 to 48 hours (JAFFE et al., 1969; KAMIENSKI and MURPHY, 1971).

Bulletin of Environmental Contamination & Toxicology,
Vol. 13, No. 6 © 1975 by Springer-Verlag New York Inc.

Experimental

Animals: Adult, male, Charles River mice weighing between 25-35 g were used. The animals were housed in air-conditioned rooms and supplied with food and water ad libitum. Single doses of 400 mg/kg of piperonyl butoxide were given by intraperitoneal injection of a 400 mg/ml solution in corn oil at 1, 24, or 48 hr before sacrifice.

Liver incubation procedures: Mice were sacrificed by cervical dislocation and their livers quickly removed, blotted and weighed. Livers were homogenized at 0-4°C in 4 volumes of a solution containing 1.15% KCl and 0.25% nicotinamide. The homogenates were kept in an ice bath until used. All incubations were performed within two hours of sacrifice. The incubation procedure was modification of the method of HITCHCOCK and MURPHY (1971). Incubation mixtures contained 80 mg liver in a solution 40 mM in sodium phosphate buffer (pH 7.6), 1.4 mM in nicotinamide adenine dinucleotide phosphate and 3.8 mM in glucose-6-phosphate of total volume of 2 ml. Following a 5-minute preincubation period, 400 nmoles of parathion were added as a 0.1 M solution in absolute ethanol by means of a Hamilton 10 µl syringe. The mixtures were incubated for 30 min in a reciprocal water bath shaker (120 oscillations/min). Standards for analysis were carried through an identical incubation except a heat-inactivated liver homogenate was added at the start of the incubation period. Appropriate tissue and reagent blanks were also included. After 30 min, a 0.1 ml aliquot of each incubation mixture was removed for analysis for paraoxon content by the anti-cholinesterase method. The remainder was analyzed for paraoxon and parathion by the gas chromatographic method. All incubations were performed in duplicate.

Gas chromatographic analysis for parathion and paraoxon: The enzyme activity was terminated by placing the flasks in boiling water for 2 min. The mixtures were then extracted twice by shaking for 1 hr with 5 ml portions of diethyl ether. The ether was decanted after chilling to -20°; the freezing step precipitates some extraneous ether-soluble material. Extracts were taken to dryness in air, and then taken up in 0.5 ml of hexane and analyzed by gas chromatography.

Analyses were performed using a 5-foot, 4 mm i.d. glass column of 5% OV-101, a methyl silicone resin, on 80/100 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pennsylvania) preconditioned with 50 µl of hexamethyldisilazane (Analabs, North Haven, Connecticut). A Micro-Tech Mt-220 gas chromatograph equipped with a Melpar flame photometric detector (FPD) for phosphorus was used under the following conditions: column oven, 195°C; injection port, 225°C; detector, 180°C; nitrogen flow, 60 ml/min; hydrogen flow, 180 ml/min; oxygen flow, 25 ml/min; air flow, 25 ml/min. The column was primed each day with injections of 0.02M paraoxon in acetone. The retention time for paraoxon was 1.37 min, for parathion, 1.75 min.

Most ether soluble organic material extracted from the incubation mixture does not interfere with the analysis because the FPD is sensitive only to phosphorus-containing compounds. Standard curves for paraoxon and parathion were prepared for each day of analysis using samples extracted from mixtures identical to incubations except that heat inactivated liver was used. Recovery of paraoxon was over 95%, recovery of parathion was over 90%.

Anti-cholinesterase assay for paraoxon: Paraoxon concentration was measured by the procedure described by BENKE, et al (1974), using the method of ELLMAN et al., (1961) to measure cholinesterase activity. After 30 min of incubation, a 0.1 ml aliquot of the liver incubation mixture was removed, rapidly diluted to 1.0 ml with deionized distilled water, and liver enzyme activity was terminated by placing the solution in a boiling water bath for 2 min. The solution was stored at -20° prior to analysis. For analysis, the samples were thawed, appropriately diluted, and varying volumes added to the cholinesterase test system to give between 15 and 90% inhibition of bovine erythrocyte cholinesterase Type I. The appropriate amount of unknown inhibitor solution was added to a solution of 5 µg cholinesterase in 0.1 M phosphate buffer (pH 8.0) to yield a total volume of 5 ml. The tubes were allowed to stand at room temperature for sixty minutes in the absence of substrate. The Ellman reagent, 0.54 µmoles of 5,5'-dithiobis-2-nitrobenzoic acid, was added in the cold, followed by 5 µmoles of the substrate, acetyl thiocholine iodine. Enzyme activity was measured as the change in absorbance at 412 nm during a 30 min incubation at 27°C. The amount of paraoxon present in the incubation mixtures was determined by comparing the inhibition with that resulting from the known concentrations of paraoxon present in the standards.

Analyses of results: The gas chromatographic and anti-cholinesterase methods of analysis for paraoxon formation were compared by calculating the correlation coefficients and average variation between results of analyses of duplicate incubates assayed by the same method, and the correlation coefficients for the activity of each liver as determined by the two different analytical methods. The significance of the differences between the means for control and treated animals was evaluated by the two-tailed t-test (DIXON and MASSEY, 1969).

Results and Discussion

The results obtained in incubations of parathion with livers of control and piperonyl butoxide treated mice are shown in the Table.

A significant decrease in paraoxon production relative to control was observed in incubations with livers of mice sacrificed 1 hr after treatment with piperonyl butoxide using both gas chromatographic and anti-cholinesterase methods. There was a small increase in paraoxon formation relative to control in mice sacrificed 24 hr after pretreatment; this increase became statistically significant by 48 hr. Parathion disappearance, representing total metabolism, was significantly less than control in livers obtained 1 hr after pretreatment,

and significantly greater than control at 24 hr. These observations are consistent with those of JAFFE et al (1969) and KAMIENSKI and MURPHY (1971).

There were small differences in the mean values for paraoxon formation when the gas chromatographic and anti-cholinesterase methods were compared for each group of animals. However, these differences were not statistically significant for any group of animals ($p>.05$). The variations between the two methods, which overall amount to about 10%, are within the likely error in standardization of the gas chromatograph detector response and the cholinesterase inhibition curves.

The correlation coefficient for results of analyses for paraoxon of duplicate incubations with tissue from the same liver by gas chromatography was $r = 0.86$. The mean difference between analysis of duplicates, expressed as the percentage of the mean value for the pair, was $10.3 \pm 1.4\%$. Using the anti-cholinesterase method, the correlation coefficient between duplicate incubations was $r = 0.87$, with a mean difference of $12.6 \pm 1.8\%$. Thus, the reproducibility of the two methods was about equal. The correlation coefficient between analyses of the same incubate by both methods was $r = 0.91$. The correlation coefficient for analyses of duplicate incubations for parathion by gas chromatography was $r = 0.95$.

The limit of detection of paraoxon by the anti-cholinesterase method, using the work-up procedure described here, is 0.5 nmoles in the total incubate, while the described procedure for the gas chromatography was reliable only to about 1 nmole. The anti-cholinesterase method could be made considerably more sensitive by increasing the size of the aliquot. However, in practice, and in the presence of large amounts of parathion, reliable detection of metabolism by both methods appears to be limited by paraoxon formed by non-enzymatic oxidation (in inactivated tissues) to greater than 2 nmoles in an incubate. This interference can be minimized by lowering phosphorothionate levels.

The anti-cholinesterase method was more rapid and convenient than the gas chromatographic method. However, with the latter procedure it is also possible to measure the total amount of parathion metabolism in the same incubate by measuring the residual parathion. Studies of the metabolism of phosphorothionate insecticides in vitro have usually been conducted in attempts to correlate metabolic activity with toxicity data. For such purposes measurement of the rate of total degradation of the parent compound in addition to measurements of specific metabolites permits the detection of degradation by pathways not known or specifically measured. The difference between the sum of paraoxon found and parathion recovered and parathion originally added to the incubate represents metabolism by other pathways, including cleavage of p-nitrophenol and perhaps dealkylation and binding. The production of p-nitrophenol has been measured in a series of parallel experiments and found to account for most of the difference.

TABLE

Metabolism of parathion by whole liver homogenates from male mice as determined by gas chromatographic and anti-cholinesterase assay methods.

Treatment (number of animals)	Paraoxon,[a] (anti-cholinesterase assay)	Paraoxon,[a] (gas chromatographic assay)	Parathion,[b] (gas chromatographic assay)
corn oil,[c] (9) 1 hr	27.3 ± 1.7	24.0 ± 1.0	260 ± 5
piperonyl butoxide,[d] (10) 1 hr	15.7 ± 0.8**	14.5 ± 0.5**	291 ± 6 **
piperonyl butoxide,[d] (10) 24 hr	30.5 ± 1.1	27.2 ± 1.3	241 ± 5 **
piperonyl butoxide,[d] (5) 48 hr	33.0 ± 1.8*	29.6 ± 2.4*	250 ± 11
boiled liver,[e] (10)	0	0	400 ± 11

[a] nmoles/80 mg liver/30 min., mean ± SEM
[b] nmoles remaining in incubate, mean ± SEM
[c] 1.0 ml/kg
[d] 400 mg/kg as 400 mg/ml in corn oil
[e] individual incubations with samples of heat inactivated liver homogenates

** significantly different from control ($p<0.01$)
* significantly different from control ($p<0.05$)

Acknowledgements

This research was supported by Training Grant ES 00045 and Research Grants ES 00084 and ES00002 from the National Institute of Environmental Health Sciences, U.S. Department of Health, Education and Welfare.

References

BENKE, G., K.L. CHEEVER, F.E. MIRER and S.D. MURPHY: Tox. Appl. Pharmacol. 78, 97 (1974).
BOWMAN, M.C. and M. BEROZA: J. Ass. Off. Anal. Chem. 53, 499 (1970).
DAVISON, A.N.: Nature, 174, 1056 (1954).
DIGGLE, W.N. and J.C. GAGE: Nature, 168, 998 (1951).
DIXON, W.A. and F.J. MASSEY: Introduction to Statistical Analysis, McGraw Hill, New York, 1969.
ELLMAN, G.L., K.D. COURTNEY, V. ANDRAS, JR. and A.M. FETHERSTONE: Biochem. Pharmacol. 7, 88 (1961).
GAGE, J.C.: Biochem. J. 54, 426 (1953).
HITCHCOCK, M. and S.D. MURPHY: Toxicol. Appl. Pharmacol. 19, 37 (1971).
HOLLINGWORTH, R.M.: J. Ag. Food Chem. 17, 987 (1969).
JAFFE, H., K. FUJII, H. GUERIN and S.S. EPSTEIN: Biochem. Pharmacol. 18, 1045 (1969).
JAGLAN, P.S., R.B. MARCH, F.A. GUNTHER: Anal. Chem. 41, 1671 (1969).
JAGLAN, P.S., R.B. MARCH, T.R. FUKOTO and F.A. GUNTHER: J. Ag. Food Chem. 18, 809 (1970).
JOHNSEN, R.E. and P.A. DAHM: J. Econ. Entom. 59, 1437 (1966).
KAMIENSKI, F.X. and S.D. MURPHY: Toxicol. Appl. Pharmacol. 18, 883 (1971).
MURPHY, S.D. and K.P. DUBOIS: J. Pharmacol. Exp. Ther. 124, 194 (1958).
MURPHY, S.D.: Proc. Soc. Exp. Biol. Med. 123, 392 (1966).
NEAL, R.A.: Biochem. J. 183-91 (1967).
NAKATSAGAWA, T., N.M. TOLMAN and P.A. DAHM: Biochem. Pharmacol. 17, 1517 (1968).
RAO, S.L.N. and W.P. MCKINLEY: Can. J. Biochem. 47, 1115 (1969).
VUKOVICH, R.A., A.J. TRIOLO and J.M. COON: J. Pharmacol. Exp. Ther. 178, 395 (1971).