A Sensitive Procedure for Urinary *p*-Nitrophenol Determination as a Measure of Exposure to Parathion

JOSEPH W. ELLIOTT,[1] KENNETH C. WALKER,[2] ARCHIE E. PENICK, and WILLIAM F. DURHAM

Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Wenatchee, Wash.

A sensitive procedure has been developed for the determination of *p*-nitrophenol in urine. The major variation from earlier published procedures involves extraction with acetonitrile to remove interfering impurities. The present method allows determination of as little as 10 γ of *p*-nitrophenol and permits the use of urine samples as large as 100 ml.

THE ACTIVITY OF THE ORGANIC phosphorus insecticides in inhibiting the enzyme cholinesterase is well known. Measurement of blood cholinesterase level has been widely used as a measure of exposure of workers and others to these agents. However, there has been a need for a more sensitive measure of exposure. In certain instances, particularly in cases of multiple exposure, it would be an advantage to have a test which is specific for a certain compound or for a certain restricted group of compounds. Urine samples are easier and less painful to acquire than blood samples requiring venipuncture, particularly where a number of tests may be made on the same person over the course of a season's work. The determination of urinary *p*-nitrophenol as a measure of exposure to those organic phosphorus compounds, including parathion and EPN, which yield this material on hydrolysis has been investigated.

There are in the literature a number of methods for the determination of *p*-nitrophenol in urine. All involve the reduction of *p*-nitrophenol to *p*-aminophenol, which then is coupled with a phenol to form indophenol blue. They differ as to whether or not hydrolysis is considered to be necessary and as to methods for extraction and purification. All suffered from the limitation that quantities of urine greater than 10 ml. could not be used because of interfering substances. In addition, with some of the methods, recovery from urine of known added amounts of *p*-nitrophenol was erratic.

The present paper points out that hydrolysis is a necessary step in the procedure. An additional cleanup step involving use of acetonitrile brings about much more complete separation of *p*-nitrophenol, from interfering impurities than was possible with earlier methods and thus makes possible the use of large quantities of urine (up to 100 ml.).

[1] Present address, Fish and Wildlife Service, U. S. Department of Agriculture, Entiat, Wash.

[2] Present address, Pesticide Chemicals Research Laboratories, U. S. Department of Agriculture, Yakima, Wash.

Procedure

A sample of urine (not more than 100 ml. and containing not less than 10 γ of *p*-nitrophenol) is placed in a 500-ml. Erlenmeyer flask fitted with a water-cooled condenser. Twenty milliliters of concentrated hydrochloric acid are added per 100 ml. of urine, and the mixture is boiled gently for 1 hour. The hydrolyzed urine is cooled and extracted with 60 ml. of ethyl ether–benzene (20 to 80) by shaking for 5 minutes. The water phase is aspirated off and sufficient anhydrous sodium sulfate (about 5 to 10 grams) added to congeal any residual emulsified aqueous phase and to dry the ether-benzene.

Fifty milliliters of the organic phase are transferred to a 250-ml. Erlenmeyer flask or glass-stoppered bottle and shaken for 5 minutes with 5 ml. of 20% sodium hydroxide. The organic layer is aspirated off, and the sodium hydroxide is poured into a 15-ml. glass-stoppered centrifuge tube. Any accompanying ether-benzene is aspirated off and residual amounts are finally removed by a gentle air stream. The previous container is rinsed with 5 ml. of acetonitrile, and the rinsings are carefully added to the glass-stoppered centrifuge tube containing the sodium hydroxide.

The centrifuge tube is stoppered and shaken for two approximately 1-minute periods. The tube is centrifuged briefly to effect a clear separation of the layers and the sodium hydroxide phase is aspirated off.

Five milliliters of 3*N* ammonium hydroxide and 1 ml. of *o*-cresol reagent (2% in 0.2*N* sodium hydroxide) are added and mixed. The solution should be observed at this time (prior to reduction) for blue color which would indicate the presence of amino compounds. One milliliter of freshly mixed titanium trichloride reagent (1 ml. of 20% titanium trichloride to 16 ml. of water) is then added. The tube is immediately shaken for about 15 seconds or until the dark precipitate disappears. Thirty minutes is allowed for full development of the indophenol blue color. Once developed, the color is stable for several hours. The tubes are then centrifuged for 2 to 5 minutes or until optically clear. The absorbance of the colored solution is then read against a reagent blank at a wave length of 620 mμ. The *p*-nitrophenol content is determined by calculation using the absorptivity obtained in the usual manner from carrying known quantities of *p*-nitrophenol through the complete procedure (Table I).

Results

The effects of varying certain of the prescribed conditions in the method were measured. Among the items studied were:

Hydrolysis. *p*-Nitrophenol may be present in human urine in either the free form or conjugated with glucuronic, sulfuric, or acetic acid (*5*). The conjugated *p*-nitrophenol must be converted to the free form before it can be successfully carried through the series of extractions and the color development step described above. In almost every instance, acid hydrolysis of urine samples from persons exposed to parathion gave higher *p*-nitrophenol values than corresponding aliquots which were not hydrolyzed. An additional advantage of acid hydrolysis was that troublesome emulsions were less frequently encountered when extractions with organic liquids were carried out following this procedure than when extractions were made from neutral or alkaline media. In a limited series of samples, it appeared that differences between hydrolyzed and nonhydrolyzed samples were smaller for samples of higher *p*-nitrophenol content. This may be an indication that, at higher levels of parathion exposure, the ability of the organism to detoxify *p*-nitrophenol by conjugation is exceeded.

Table I. Absorptivity and Recovery of Known Amounts of p-Nitrophenol[a]

p-Nitrophenol Content	Direct Color Development		Full Procedure		
	Absorbance	Absorptivity[b] × 10^2	Absorbance	Absorptivity[b] × 10^2	Recovery, %
10	0.150	1.50	0.115	1.15	75
20	0.305	1.53	0.230	1.15	75
30	...	...	0.335	1.12	73
40	...	...	0.465	1.16	75
50	0.790	1.58	0.535	1.07	70
100	1.55	1.55	1.10	1.10	71
Mean		1.54		1.13	73.2
Standard error of mean		0.017		0.015	

[a] Determinations reported in columns 4 and 5 were carried through entire procedure described above. Determinations in columns 2 and 3 involved color development only.
[b] Calculated using concentration expressed as micrograms per 12 ml.

The results noted could be due to excretion of free parathion in the urine following high-level exposure. However, no parathion could be detected either by chemical or by enzymatic methods in urine from persons known to have large parathion exposure and who were, at the time of study, excreting high levels of *p*-nitrophenol.

No advantage was noted in extending hydrolysis time up to 4 hours or in increasing acid strength beyond that recommended above. A 1-hour hydrolysis period gave somewhat better results than did shorter times.

In contrast to results with human urine, hydrolysis brought about no increase in recovery of *p*-nitrophenol from the urine of parathion-dosed rats. This variation is thought to be a species difference, as the dosage levels in the two instances were of about the same order of magnitude. The mature male rats had been fed a diet containing 50 p.p.m. of parathion, which determines an oral dosage of about 2 mg. per kg. per day. The daily exposure of orchard spraymen to parathion has been estimated to be about 8.9 mg. per kg. (*2*), chiefly by the dermal route.

Extraction. The use of ion exchange resins, including Amberlite resins IR4-B, IR45, IR50, IR120, IR400, and IR401 (Rohm & Haas Co., Philadelphia, Pa.) to separate *p*-nitrophenol from interfering substances in urine was investigated. A number of resins were found which would absorb *p*-nitrophenol. However, of the resins tested, none would selectively adsorb *p*-nitrophenol without interfering substances and allow quantitative recovery of the adsorbed *p*-nitrophenol. Better purification and better recovery were obtained with solvent extraction than with any resin tested.

All three of the extraction steps involved, including ether-benzene from aqueous hydrochloric acid; aqueous sodium hydroxide from ether-benzene; and acetonitrile from aqueous sodium hydroxide, are almost quantitative and give recoveries of known added amounts of *p*-nitrophenol between 80 and 100%. The latter transfer (from sodium hydroxide to acetonitrile) requires vigorous shaking to mix the phases and it is necessary that the alkali be concentrated (20% or above) so that the acetonitrile will not extract water as well as *p*-nitrophenol.

Recovery. The relationship between *p*-nitrophenol content and absorbance of the indophenol blue product at 620 mμ as measured in a Beckman Model B spectrophotometer is shown in Table I. The agreement of the absorptivity values shows that the reaction follows Beer's law closely between 10 and 100 γ of *p*-nitrophenol. At higher values of *p*-nitrophenol content (above 250 γ), the relationship between *p*-nitrophenol content and absorbance is no longer linear. However, it is possible to obtain fairly accurate estimations of *p*-nitrophenol content of solutions too deeply colored for accurate direct reading (above absorbance of 1.55) by dilution with acetonitrile.

The column in Table I headed Direct Color Development represents values obtained by adding known amounts of *p*-nitrophenol directly to the tubes in which the final color reaction took place so that no recovery of *p*-nitrophenol by extraction was involved. The absorptivities are higher (average 0.0154) than those obtained when the nitrophenol was extracted from the urine (average 0.0113). The average absorptivity, corrected for aliquots taken, of the Full Procedure determination, is only 73% as large as that of solutions in which the color was developed directly. An average loss of 27% of the added *p*-nitrophenol is incurred in carrying out the full procedure. While desirable, it is not necessary that 100% recovery of material be possible in analytical methods of this type. It is, however, essential that a constant percentage be attained. The present method yields a rather constant recovery of from 70 to 75%. No samples of known amounts of conjugated *p*-nitrophenol were available, so no studies were made of the completeness of hydrolysis. Therefore, it must be understood that in actual practice the recovery of total (including both free and conjugated) *p*-nitrophenol from urine may be slightly lower because of possible incomplete hydrolysis. However, the samples referred to in Table I under Full Procedure were carried through the hydrolysis step to allow for any losses incurred during these manipulations.

Specificity. The specificity of the *p*-nitrophenol analysis would be improved if one could determine whether any of the final colored complexes represented *p*-aminophenol. *p*-Aminophenol might occur in the urine as a natural metabolite of parathion following in vivo reduction of parathion (*1*), of *p*-nitrophenol (*3*), or as a biotransformation product of certain common drugs, such as acetophenetidin. Distinction between amino and nitro compounds is not possible by previously published procedures. However, by simply adding the color-producing reagent to the reaction mixture before carrying out the reduction step, it is possible to determine pre-existing amino compounds. (Any blue color formed after addition of *o*-cresol and before addition of titanium chloride could be ascribed to amino compounds.) Tests have shown that the nitro compounds give the same eventual depth of color regardless of whether the *o*-cresol reagent is added before or after reduction. In none of the samples analyzed have the authors noted the presence of amino compounds, following the recommended cleanup procedure.

The authors have found it convenient to express *p*-nitrophenol excretion in terms of parathion equivalent. This conversion can, of course, be made by multiplying *p*-nitrophenol excretion by a factor of 2.09.

Storage. Formalin (2 to 3 drops per 100 ml. of urine) is a good preservative for urine samples collected for *p*-nitrophenol determination. Refrigeration is also recommended where samples are to be kept for several days before analysis.

Unpreserved human urine samples showed losses of *p*-nitrophenol, on standing, ranging from 1 to 24% per day. A more rapid loss of *p*-nitrophenol occurred in those urine samples with stronger odor of ammonia.

Discussion

Preliminary tests indicated that, with minor modifications, this procedure could be made applicable to other organic phosphorus insecticides which yield substituted nitrophenols on hydrolysis. Among the compounds in this category which have been considered are Chlorthion [*O*-(3-chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate] and Dicapthon [*O*-(2-chloro-4-nitrophenyl)*O,O*-dimethyl phosphorothioate]. The former compound would yield 3-chloro-4-nitrophenol and the latter material would give rise to 2-chloro-4-nitrophenol.

A number of common insecticides and drugs have been tested for possible interference in this procedure. Of the materials tested, the only substance which interfered in the analysis was dinitro-*o*-cresol (DNOC). This finding is not surprising in view of the similarity between the dinitro-*o*-cresol molecule (which is really a disubstituted *p*-nitrophenol) and that of *p*-nitrophenol. Dinitro-*o*-cresol produces a yellow color in the reaction which has the effect of a high blank value. The magnitude of this interference is not very great even at low *p*-nitrophenol values and is undetectable at levels above 25 γ.

Studies of exposed workers carried out during the 1958 spray season have shown that, when measured by the present method, urinary *p*-nitrophenol excretion is significantly more sensitive than blood cholinesterase in measuring absorption of parathion. This statement is true even under optimal conditions for usage of blood cholinesterase values—when the individual's pre-exposure enzyme level is known. The results of these studies will be reported in detail elsewhere.

The present method for determination of urinary *p*-nitrophenol content can be carried out in about the same period of time as required for blood cholinesterase determination by the Michel procedure (*4*). It is not proposed that measurement of urinary *p*-nitrophenol be substituted for blood cholinesterase determination in all cases involving exposure to parathion or EPN. In some instances, one or the other analysis may be preferable; in other cases, it may be informative to carry out both tests.

Literature Cited

(1) Ahmed, M. K., Casida, J. E., Nichols, R. E., J. AGR. FOOD CHEM. **6**, 740 (1958).
(2) Batchelor, G. S., Walker, K. C., *Arch. Ind. Hyg. Occupational Med.* **10**, 522 (1954).
(3) Gardocki, J. F., Hazleton, L. W., *J. Am. Pharm. Assoc., Sci. Ed.* **40**, 491 (1951).
(4) Michel, H. O., *J. Lab. Clin. Med.* **34**, 1564 (1949).
(5) Williams, R. T., "Detoxication Mechanisms," p. 136, Wiley, New York, 1949.

Received for review July 1, 1959. Accepted October 28, 1959.

SOIL FUMIGANT DETERMINATION

Extraction and Determination of Ethylene Dibromide in Soils

J. J. JURINAK, A. L. BROWN, and P. E. MARTIN

Department of Soils and Plant Nutrition, University of California, Davis, Calif.

A method utilizing vacuum distillation and catalytic oxidation is described for the determination of ethylene dibromide in soils. Recovery is reported in the range of about 82 to 0.2 mg. of ethylene dibromide per 50 grams of soil. Moisture and organic matter affect recovery.

IN STUDIES INVOLVING penetration and phytotoxic effects of ethylene dibromide (EDB) in soils it is necessary that an analytical technique be developed to determine the low concentrations of the fumigant usually encountered in soil studies.

Previous studies were either lengthy and prone to both oxidizing and reducing side reactions (*1*), or lacking in sensitivity at low concentrations (*4*).

This investigation is an attempt to develop a simple but sensitive technique for the determination of ethylene dibromide in soils. This determination is carried out in three stages: recovery of ethylene dibromide from the soil sample, conversion of ethylene dibromide to inorganic bromide, and determination of inorganic bromide.

Reagents and Apparatus

2,4,5,7-Tetrabromofluoroscein, sodium salt (eosin Y), 0.1% solution.

The catalytic oxidation of ethylene dibromide is based on a modification of the method described by Lubatti and Harrison for the determination of methyl bromide (*3*). The combustion or reaction chamber is shown in Figure 1. Two 15-mm. lengths of 20-gage platinum wire are introduced into the stopper, *B*, by means of capillaries, *C* and *C'*; and sealed in airtight with Apiezon W wax. To the lower ends of the thicker platinum wires are connected 16 mm. of coiled 30-gage platinum wire which serves as the oxidizing surface. During the catalytic oxidation, the leads of *C* and *C'* are connected to an electrical circuit through a variable transformer. In this study, 5 volts were sufficient to bring the filament to a bright red glow. The 14/35 taper attached to *B* is used to connect the reaction chamber to the vacuum manifold for the evaluation of the flask. The vacuum system used maintained a pressure of 1 to 5 microns as measured with a McLeod gage.

Procedure

The purity of ethylene dibromide used in the study was determined by adding, with a micropipet, known amounts of ethylene dibromide to flask *A*, then immediately immersing the flask in a dry ice–acetone bath. To the frozen system were then added 2 ml. of 0.8*N* sodium hydroxide and 1.5 ml. of 30% hydrogen peroxide. Stopper *B* was placed on the flask and the system was completely evacuated while still immersed in the freezing bath. The system was then removed from the vacuum manifold, was thawed, and sufficient voltage was placed across the filament to produce a bright glow. The combustion period consisted of alternate closing and opening the circuit at 1-minute intervals, to prevent overheating, for about 15 minutes. Stopper *B* was then removed and rinsed. The solution in flask *A* was then directly titrated with either 0.01*N* or 0.025*N* silver nitrate using a microburet and following the eosin Y method of Kolthoff and Sandell (*2*).

At two concentration levels of 21.7 and 43.4 mg. of ethylene dibromide added to the reaction flask successive determinations indicated a recovery of 96.3, 96.5, and 96.7% and 95.5, 96.6, and 96.7%, respectively. Hence under