synergist for carbaryl than the technical grade and the components were even less effective. The high synergistic activity of piperonyl butoxide and the sulfoxide synergist analogs but not of safrole or dihydrosafrole for pyrethrum toxicity to houseflies was as anticipated (*10, 14, 16, 21, 22*). Pyrethrum synergism was optimal with the components of the 2-octylsulfinyl analog but was also high with many other analogs. With both the sulfides and the sulfones, pyrethrum synergism decreased in the order of 1 > 2 > 3 for the position of sulfur attachment to the propyl group. Of particular interest was the finding that the more polar component (B or B′) was a more effective pyrethrum synergist than the less polar component (A or A′) of the 2-octylsulfinyl and, especially, the 1-octylsulfinyl compounds. The isomeric configuration about the sulfoxide grouping and the asymmetric carbon of the propyl grouping, therefore, influences the activity for synergism of pyrethrum toxicity.

In tests made under conditions somewhat similar to those used in this study, myristicin has been found to have a high degree of synergism for carbaryl but not for pyrethrum toxicity (*11*).

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INSECTICIDE RESIDUES IN URINE

Determination of Urinary *p*-Nitrophenol by Thin-Layer Chromatography and Phosphorimetry

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Human urine is analyzed for *p*-nitrophenol, a major metabolite of parathion. After acid hydrolysis, the urine is extracted with ether. The ether extract is then cleaned up by thin-layer chromatography. A urine blank is employed to account for the remaining urine background. The final measurement is by the extremely sensitive technique of phosphorimetry. The time required for the entire procedure is only 40 minutes and only 5 ml. of urine is required for the analysis of urine samples containing at least 0.01 μg. of *p*-nitrophenol. The average recovery of *p*-nitrophenol in the concentration range of 0.28 to 142 μg. per 100 ml. of urine is 88%. A relative standard deviation of 2.5% is obtained for a urine specimen containing 7.0 μg. of *p*-nitrophenol per 100 ml. of urine.

ONE of the most commonly used organophosphorus insecticides is parathion. Because of its widespread use and high toxicity, sensitive and accurate analytical techniques are needed in many agricultural and clinical laboratories to protect agricultural workers and consumers. The methods must be not only highly sensitive but also simple enough to permit their use for routine analyses.

The widely used technique of measuring blood cholinesterase activity has numerous faults when applied to methods for monitoring human parathion exposure (*3, 6*). The cholinesterase method, however, does have the advantage of measuring the effect of parathion on enzyme activity. The direct spectrophotometric measurement of *p*-nitrophenol excretion in urine is preferred (*1, 5, 8*) in many instances. These

methods still required about 10 μg. of *p*-nitrophenol for an accurate analysis. The direct measurement of *p*-nitrophenol excretion does, however, have several limitations. For example, the measurement of the metabolite does not differentiate between the different toxicities of various forms of parathion—i.e., the oxidized form, paraoxon, is much more toxic, although each form still results in the excretion of *p*-nitrophenol. Also the measurement of *p*-nitrophenol does not differentiate between toxicities resulting from variations in routes of absorption—e.g., dermal exposure is less toxic than respiratory exposure. In spite of these limitations, a method of monitoring low concentrations of *p*-nitrophenol in urine is still of great value to the research chemist, for the monitoring of *p*-nitrophenol in the urine of small animals as well as in human urine.

The principles of phosphorimetry have been considered by Keirs, Britt, and Wentworth (*4*), Parker and Hatchard (*7*), and Winefordner and Latz (*9*). Freed and Salmre (*2*) and Winefordner and Latz (*9*) showed that phosphorimetry could be used for determining constituents containing conjugated structures in biological fluids. Winefordner and Latz (*9*) also made a thorough study of the phosphorescent constituents in blood and urine and applied phosphorimetry to the accurate determination of low concentrations of aspirin in blood. Winefordner and Moye (*10*) have shown that phosphorimetry can be used in conjunction with thin-layer chromatography to determine small amounts of the major alkaloids in tobacco. Winefordner and Tin (*11*) obtained excellent sensitivities using phosphorimetry to determine cocaine and atropine in urine. However, because the urine was not hydrolzyed, the problem of significant urine background was not encountered. Because small quantities of *p*-nitrophenol in urine were measured in the studies described in this manuscript, it was necessary to include a hydrolysis step (*1*).

Experimental

Apparatus. All phosphorimetric measurements were taken with the Aminco Bowman Spectrophotofluorometer (No. 4-8202) with the phosphoroscope attachment (No. C27-62140, American Instrument Company, Inc., Silver Spring, Md.). The mercury-xenon lamp (No. 416-993) was used for all quantitative measurements, while the xenon lamp (No. 416-992) was used to record all spectra. The quantitative measurements were made with the following slit program: A, 3 mm.; B, 4 mm.; C, 4 mm.; D, 3 mm.; and E, 3 mm. The spectra were recorded with the following slit program: A, 3 mm.; B, 0.5 mm.; C, 0.5 mm.; D, 3 mm.; and E, 0.5 mm.

All spectra were recorded with a Moseley X-Y recorder (No. 135-A F. L. Moseley Co., Pasadena, Calif.).

Reagents and Materials. Merck silica gel G (Brinkmann Instrument Co., Cantiague Road, Westbury, N. Y.) was used for all thin layers. Pittsburgh microscope slides (Fisher Scientific Co.), 75 × 25 mm., were used for thin-layer supports. Reagent grade oxalic acid, hydrochloric acid, toluene, and diethylamine were used. Technical grade ethyl ether was redistilled at a reflux ratio of 20 to 1 using a 5-foot vacuum jacketed, helices packed column (*11*). Absolute ethanol (Union Carbide) was purified in the same way as the ether. The *p*-nitrophenol (Eastman) was recrystallized from water.

For calibration of the phosphorimeter a toluene stock solution containing 0.2 ml. of toluene per 100 ml. of ethanol solution was prepared. It was diluted tenfold with ethanol to prepare the toluene standard. Both were stored at 0° C. in a refrigerator in screw cap bottles.

Procedure

Treatment of Urine. To 90 ml. of urine, 10 ml. of concentrated HCl is added. A minimum of 5 ml. of urine is necessary to perform the following studies. The urine is refluxed for one hour and then stored in a refrigerator. At least 6 ml. of this urine solution is put into a 12-ml. centrifuge tube and spun down at high speed (6000 r.p.m.). Then exactly 5 ml. is carefully drawn off with a pipet for analysis.

Preparation of Thin Layer. The silica gel G contains organic contaminants which give a phosphorescent background. To remove this background, the silica gel is heated at 700° C. for at least 12 hours. This does not destroy the normal chromatographic or physical properties of the silica gel, although a slight pink coloration may become evident.

Fourteen thin layers are simultaneously made by placing 14 75 × 25 mm. microscope slides on a 20 × 20 cm. glass plate. A dish 2 cm. deep is made by applying a strip of masking tape to the edges of the plate. In a graduated Erlenmeyer flask with an aluminum-wrapped cork, enough 0.1*M* oxalic acid is added to 150 ml. of the cleaned silica gel to make a 150-ml. volume slurry. The slurry should be shaken thoroughly to ensure that no dry clumps of silica gel remain.

The slurry is immediately and slowly poured onto the plate, care being taken to position the plate on a perfectly level surface to ensure even settling of the silica gel. The thin layers are room-dried, giving a layer thickness of about 2 mm. when dry. The masking tape is then stripped off, and the layers are activated at 115° C. for at least one hour, stored at room temperature and humidity (60%), and used without reactivation. Care should be taken to prevent dust contamination of the activated plates.

The thin layers are separated with a razor blade, and about 2 mm. is trimmed off three edges and 4 mm. off the top. This allows easier handling.

Extraction of Urine. Only one extraction of the acidified urine is needed to recover essentially all the *p*-nitrophenol. Urine, 5-ml., is pipetted into a snap-on polyethylene capped vial, 6 ml. of ether is added, and the vial is shaken vigorously for 5 minutes. The aqueous phase is removed with a hypodermic syringe having an extra long needle. Water beads inside the vial do not interfere. After evaporation of the ether solution to about 2 ml. in vacuo, the ether solution is applied to the activated thin layer.

Application of Urine Extract to Thin Layer. A capillary pipet is made from eleven open-end capillary melting point tubes. They are mounted side by side on a small block of polystyrene and have their ends polished smooth to prevent chipping of the thin-layer surface.

The ether is applied as a band 1 cm. from the bottom of the thin layer, using two 1-ml. portions of ether to rinse the vial. Care must be taken to avoid touching the pipet to the sides of the vial, where water may be picked up and harmfully applied to the thin layer.

Table I. Background Phosphorescence of Urine from Eight Donors

Urine Specimen No.	Acidic Reading[a,b]	Basic Reading	Ratio[c]	Δ[d]
1	21	68	3.24	0.15
2	34	110	3.24	0.15
3	26	81	3.12	0.03
4	62	200	3.22	0.13
5	54	170	3.14	0.05
6	31	85	2.75	0.34
7	40	110	2.76	0.33
8	32	105	3.30	0.21
		Av.	3.09	
		Std. dev.	0.22	
		Rel. std. dev., %	7.1	

Average urinary background expressed as equivalent *p*-nitrophenol concentration in micrograms per 100 ml., 1.3.

[a] The photometer coarse sensitivity scale was 0.001. Fine sensitivity was set as explained in calibration procedure but was always close to 40. Internal gain setting of photometer was wide open.
[b] Corrected for thin-layer–solvent background.
[c] Ratio of basic reading to corrected acidic reading.
[d] Deviation from mean value.

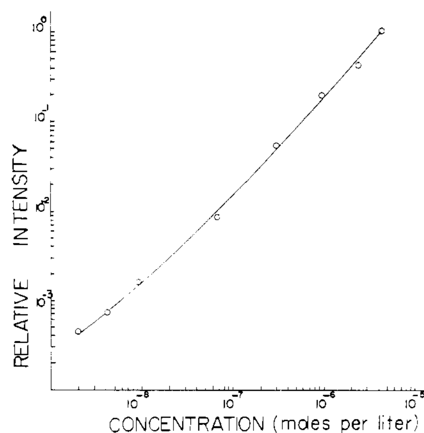

Figure 1. Analytical curve for p-nitrophenol in basic ethanol-ether solution

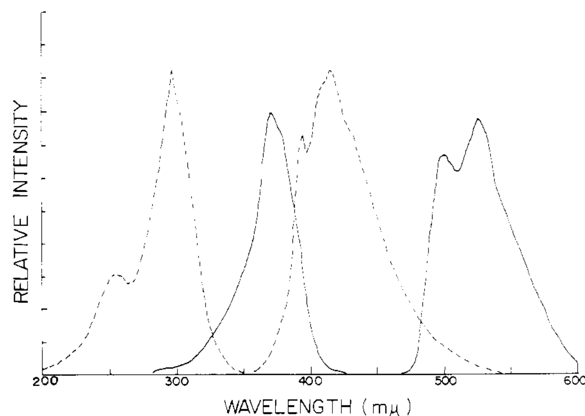

Figure 2. Phosphorescence excitation and emission spectra for urine background (dotted line) and p-nitrophenol (solid line) in basic ethanol-ether solution (meter multiplier 0.003, p-nitrophenol concentration $5.1 \times 10^{-5}M$)

With a blast of hot air from a hair dryer close to and focused on the area of application, the time required to apply one sample is about 5 minutes.

Development of Thin Layer. A circular jar 11 cm. high and 8 cm. in diameter is used as a developing tank, with a glass plate for a top. Filter paper is used to line the tank. The thin layer is developed by the ascending technique. A layer of ether, about 0.5 cm. in depth, is used as the developing solvent. Development is continued until the ether has moved exactly 5.5 cm. above the origin, as can be noted by a scratch in the surface of the thin layer. All the thin-layer material is scraped away with a razor blade, except the area which is 3.4 to 4.2 cm. above the origin. This should be directly above a strong blue fluorescent band which shows up clearly on the thin layer under an ultraviolet light (primarily 3650 A radiation).

Extraction of Thin Layer. The retained portion of silica gel is scraped into a capped vial, and 5 ml. of 0.1*M* HCl is added. The vial is shaken vigorously for 10 minutes, and the slurry is transferred quantitatively to a 12-ml. centrifuge tube with several 1-ml. rinsings of water and spun at high speed for several minutes. The supernatant liquid is then decanted into another vial by inverting the centrifuge tube and allowing a minute for complete drainage. Ether, 5 ml., is added to the clear liquid. This is shaken vigorously for 5 minutes. The aqueous layer is removed with a hypodermic syringe with an extra long needle, and the remaining ether is transferred with two 1-ml. washings to a 10-ml. volumetric flask and diluted to the mark with ethanol. This gives an ethanol-ether solution which freezes to a clear, rigid glass (*9*) at liquid nitrogen temperatures.

Phosphorimetric Measurement. Prior to any phosphorimetric measurement the instrument is calibrated using the standard toluene solution. The excitation wavelength is set at 270 mμ, the emission wavelength at 385 mμ, and the meter multiplier at 0.3. The fine sensitivity of the photomultiplier photometer is adjusted so that this solution gives a reading of 84. This calibration is always performed before each series of runs, although it rarely requires a change in fine sensitivity position. The wavelengths of the excitation and emission monochromator are then set at 265 and 525 mμ, respectively, for the quantitative measurements on urine.

To obtain the background phosphorescence due to normal urine—i.e., urine samples containing no *p*-nitrophenol, the ethanol-ether solutions are prepared by the procedure described for several (from different subjects) urine samples containing no *p*-nitrophenol. These solutions are slightly acidic, since the ether is saturated with 0.1*M* HCl. The phosphorescence intensity—obtained by multiplying the phosphorescence signal read on the photomultiplier photometer meter (read as % T) times the coarse meter multiplier—of the acidified ethanol-ether solution was then measured. All phosphorimetric readings are also corrected for the thin-layer solvent background. This background never exceeded 10 (i.e., 10%) on the 0.001 scale, and is obtained by running 5 ml. of distilled water through the entire procedure used for the urine samples. The ethanol-ether solutions are then made slightly basic by the addition of four small drops (0.1 ml.) of diethylamine. Phosphorimetric intensity readings are obtained for these solutions and are corrected for the thin-layer–solvent background as described above. The ratios of the corrected readings of the basic solutions to the corrected readings of the acidic solutions gave an average of 3.1 (Table I). Because *p*-nitrophenol showed negligible phosphorescence in acidic solution, it is possible to obtain a urine blank directly on the urine sample being measured by taking a reading of an acidic ethanol-ether solution, correcting it for thin-layer–solvent background, and multiplying it by 3.1.

To obtain the *p*-nitrophenol concentration in urine:

1. Obtain the phosphorescence intensity of the acidified ethanol-ether solution.
2. Subtract the phosphorescence intensity of the thin-layer–solvent blank.
3. Multiply the resultant phosphorescence intensity by 3.1 to obtain the phosphorescence intensity of the urine blank.
4. Make the ethanol-ether solution basic and measure the phosphorescence intensity.
5. Subtract the phosphorescence intensity of the thin-layer–solvent blank from the phosphorescence intensity of the basic solution.
6. Subtract the phosphorescence intensity of the urine blank obtained in item 3 from the phosphorescence intensity obtained in item 5.
7. Using the analytical curve of phosphorescence intensity *vs.* *p*-nitrophenol concentration (see Figure 1), determine the *p*-nitrophenol concentration corresponding to the phosphorescence intensity obtained in item 6.

Calculations. The *p*-nitrophenol concentration in the original urine sample (expressed as micrograms per 100 ml.) can then be calculated from the expression

$$C(\mu g./100\ ml.) = 3.08 \times 10^7\ Y,$$

where Y = concentration in moles per liter as read from the analytical curve and the factor 3.08×10^7 accounts for the dilution steps.

Precautions. All glassware must be kept immaculately clean and free from dust. Only the middle one third of the ether and ethanol distillates is used. This resulted in solvents of extremely high purity. Glassware should be cleaned with Drene shampoo, and in no case should detergent be used (*9*).

Special attention should be given to the oven for thin-layer activation. If the thin layers become contaminated in the oven, the oven should be cleaned and heated to its maximum temperature while the door is periodically opened.

The microscope slide thin layers should be separated with a sawing motion of the razor blade rather than with a chopping motion.

Discussion

Comparison of the phosphorimetric spectra of *p*-nitrophenol and the urine

Table II. Recovery of p-Nitrophenol Added to Urine

p-Nitrophenol, μg.[a]		
Added to 5 ml. urine	*Recovered from 5 ml. urine*	*Recovery, %*
7.1	6.4	90
3.5	3.1	89
1.3	1.2	92
0.71	0.61	86
0.35[b]	0.34	97
0.071	0.057	80
0.014	0.019	136
	Av.	88%[c]

[a] Three separate 5-ml. urine samples for each amount were analyzed. The average of three results is recorded. Urine used was from the same specimen.

[b] Analyzed with relative standard deviation of 2.5%.

[c] Last sample is excluded from average.

component which appeared with it on the thin layer (Figure 2) shows that nearly all of the background is eliminated by choosing the correct excitation and emission wavelengths. Even so, an average urine background equivalent to 1.3 μg. per 100 ml. of *p*-nitrophenol resulted (Table I). This can be accounted for, however, with good accuracy by using the factor of 3.1. Good recoveries and reproducibilities are obtained for extremely low *p*-nitrophenol concentrations (Table II).

Various thin-layer materials were tried including aluminum oxide G, polyamide, and neutral silica gel. Neutral silica gel gave the best separation of these but tended to bind small quantities of *p*-nitrophenol uniformly over the developed portion of the thin-layer surface. This gave appreciable losses at low *p*-nitrophenol loads. By making the thin-layer material slightly acidic, it was possible to eliminate the sites causing holdup. Using the neutral silica gel plates, it was possible to recover only about 70% of 0.35 μg. of *p*-nitrophenol by scraping the same portion. In addition, the acidified silica gel thin layer turned out to be harder and more difficult to chip and break in handling. This was especially important during sample application where the pipet comes in contact with the thin-layer surface. The thin layers were unusually thick (2 mm.) to prevent streaking of the exceptionally large amounts of urinary materials.

Because *p*-nitrophenol is much more soluble in aqueous base than in aqueous acid, it would seem that base rather than acid should be used to remove the compound from the thin-layer material. On the contrary, recoveries were extremely low when 0.1*M* NaOH was used, probably because the base did not give as finely divided particles in the ashing as did the 0.1*M* HCl. Apparently the base does not hydrolyze the $CaSO_4$ binder, and the acid does. Neutral water performed nearly as well as acid.

No attempt was made to isolate and identify the material causing the small urine background reading. It appeared only after the hydrolysis step, however, and its phosphorimetric characteristics were not like those of a host of compounds that normally appear in urine. Dialysis was attempted on the untreated urine in the hope that the background compound(s) might have come from a protein. This brought no decrease in background as compared with urine that was not dialyzed previous to hydrolysis.

A naturally decaying phosphorescing species follows the equation $I = I_0e^{-t/\tau}$, where I is the intensity at any time t, I_0 is the intensity at $t = 0$, τ is the time required for decay to 0.37 of the original intensity, and t is the time after which excitation is stopped. A plot of log intensity *vs.* time for a decaying compound gives a straight line. A second compound of comparable intensity but different τ, if in solution with the first, will produce a break in the plot of log intensity *vs.* time. To obtain this plot, the phosphorescence signal owing to the urine background was measured on the X-Y recorder after cutoff of the exciting radiation. The data from the decay curve were then transposed to semilogarithmic paper. Similar semilogarithmic plots were obtained at three different emission wavelengths on the urine background; each resulted in a straight line, indicating the probability that the background is caused by one compound.

The entire procedure, after hydrolysis, requires at longest only 40 minutes. In addition, the sensitivity of analysis is great and good reproducibility and accuracy are attained throughout the range of concentrations. The simplicity of the method should lead to the use of this technique in routine analyses. It is hoped that this method will be put to use as a routine procedure by researchers to detect *p*-nitrophenol in urine.

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