

## NEW SPRAY TESTS FOR DETECTING ORGANOPHOSPHORUS COMPOUNDS ON THIN-LAYER CHROMATOGRAMS

J. E. BARNEY II

*Midwest Research Institute, Kansas City, Mo. (U.S.A.)*

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Organic compounds containing phosphorus, such as pesticides and acids, may presently be detected on thin-layer chromatograms with several reagents. These include ferric chloride followed by sulfosalicylic acid<sup>1</sup>, iodine<sup>2</sup>, N-chloro-2,6-dibromo-*p*-benzoquinoneimine<sup>3</sup>, iodoplatinate<sup>4</sup>, silver nitrate<sup>5</sup>, fluorescein following bromine or N-bromosuccinimide<sup>6,7</sup>, palladium chloride<sup>8</sup>, and 4-methylumbelliferone following bromine<sup>9</sup>. BUNYAN<sup>10</sup> recommends bromophenol blue, bromine plus bromophenol blue, or serum-bromothymol blue plus acetylcholine bromide. All of these tests are not specific for the phosphorus-containing moiety, and interferences may be caused by a variety of phosphorus-free compounds.

The most widely used specific reagent for detecting phosphorus-containing pesticides is HANES reagent<sup>11</sup>. Many modifications of the reagent have been offered, but the most widely used appears to be a combination of ammonium molybdate, hydrochloric acid, perchloric acid, water, and acetone<sup>12</sup>. After this reagent has been sprayed on the plate the latter is exposed consecutively to infrared and ultraviolet radiation. The formation of molybdenum blue is a positive test for phosphorus compounds. Unfortunately, the background becomes blue also, and the contrast between it and the spot is often poor. Consequently, several methods have been devised for increasing the contrast<sup>13</sup>. Many organophosphorus compounds are not readily hydrolyzed to orthophosphate by HANES reagent, so other modifications have been proposed to increase the rate of hydrolysis. In one<sup>14</sup>, certain organic phosphates are hydrolyzed enzymatically by phosphatase.

The best modifications of the HANES reagent are still not satisfactory for many organophosphorus compounds. BUNYAN<sup>10</sup> has summarized the disadvantages of the reagent. The reagent is unstable and must be freshly prepared at frequent intervals. The contrast between the spots and the background is usually poor and changes rapidly with time. The sensitivity of the reagent is poor, probably because the hydrolysis reaction is incomplete.

The present investigation has led to the development of two new spray tests for detecting many organophosphorus compounds, including organophosphates, organophosphonates, organophosphoric acids, organophosphonic acids, and related thio compounds. The tests will differentiate among inorganic phosphates, organic phosphates, and organic phosphonates. They are as simple to use as the HANES reagent, and most of the reagents are stable. They will detect less than 1  $\mu$ g of most organophosphorus compounds.

## EXPERIMENTAL

*Apparatus*

Microchromatoplates were prepared according to the method described by WASICKY<sup>15</sup>. The apparatus for preparing the plates has been described elsewhere<sup>2</sup>. Standard microscope slides, 25.4 mm × 76.2 mm × 1.2 mm, were used without preliminary cleaning. A Corning Model PC-35 Pyroceram hot plate (Corning Glass Works, Corning, N.Y.) was used for heating the plates after spraying them with the hydriodic acid and ammonium persulfate reagents. The chief feature of this hot plate is an inert ceramic top which conducts heat readily. When the hot plate was set at "4", the temperature of the surface of a microscopic slide in the center of the hot plate was 250°. The samples were spotted on the plates with 10- or 50- $\mu$ l Hamilton microsyringes. All the spray reagents were applied with a small glass sprayer (No. 2C-50, available from Microchemical Specialties Company, Berkeley, Calif.). Filtered compressed air was used for aspirating the reagents from the sprayer.

*Phosphorus compounds*

The source and estimated level of purity of the phosphorus compounds, the concentrations of the solutions used in the tests and the solvents used to prepare these solutions are presented in Table I. All of these solutions were stored in glass bottles sealed with Polyseal caps. They were used as soon as possible after preparation to minimize artifacts caused by decomposition of the pesticides. Methyl- and ethylphosphonic acid were prepared by acid hydrolysis of dimethyl methylphosphonate and diethyl ethylphosphonate, while O-methyl methylphosphonic acid and O-ethyl ethylphosphonic acid were prepared by basic hydrolysis of the respective di-esters.

*Spray reagents*

All spray reagents were prepared with distilled water and reagent-grade chemicals except as noted.

*Ammonium molybdate reagent.* Dissolve 2.0 g of ammonium molybdate in a minimum amount of 1 M HCl and dilute the solution to 100 ml with 1 M HCl. This solution is stable for several months.

*Benzidine reagent.* Agitate 0.050 g of benzidine (Matheson, Coleman, and Bell) in 10 ml of acetic acid and a minimum amount of water until the benzidine dissolves. Dissolve 22.5 g of sodium acetate trihydrate in this solution and dilute it to 100 ml with water. This solution is stable for at least two weeks.

*HI reagent.* Mix 11.2 ml of 57 % hydriodic acid (free of inhibitors) and 50 ml of glacial acetic acid, and dilute this solution to 100 ml with water. This solution is stable for several months.

*Ammonium persulfate reagent.* Dissolve 5.7 g of ammonium persulfate in a minimum amount of water and dilute the solution to 100 ml with water. This solution must be prepared fresh daily.

*Methods*

These methods have been devised for detecting various classes of organophosphorus compounds on thin-layer microchromatoplates. If the methods are used with thicker glass plates, the setting of the hot plate should be raised so that the tempera-

TABLE I  
SUMMARY OF PHOSPHORUS COMPOUNDS AND SOLUTIONS

Compound	Source	Estimated purity	Concentration (mg/ml)	Solvent
Co-Kala <sup>a</sup> (O,O-diethyl O-3-chloro-4-methyl-1-oxo-2H-1-benzopyran-7-yl phosphorothioate)	Chemagro	Commercial	1.00	Methanol
DDVP (Vapona <sup>b</sup> , Dichlorvos, O-2,2-dichlorovinyl O,O-dimethyl phosphate)	Shell	m.p. = 95° 99%	1.00	Methanol
Delnav <sup>c</sup> [dioxathion, 2,3- <i>β</i> -dioxane S,S-bis-(O,O-diethyl phosphorodithioate)]	Merck	Commercial	1.00	Methanol
Dipterex <sup>a</sup> trichlorfon, O,O-dimethyl (1-hydroxy-2,2,2-trichloroethyl) phosphonate	Chemagro	100%	1.00	Methanol
Di-Syston <sup>a</sup> [disulfoton, O,O-diethyl S-2-(ethylthio)ethyl phosphorodithioate]	Chemagro	99%	1.00	Methanol
EPN (O-ethyl O- <i>p</i> -nitrophenyl phenylphosphonothioate)	du Pont	"Pure"	1.00	Methanol
Guthion <sup>a</sup> [O,O-dimethyl S-4-oxo-1,2,3-benzotriazin-3(4H)-yl-methyl phosphorodithioate]	Chemagro	m.p. = 36° Commercial	1.00	Methanol
Meta-Systox-R <sup>a</sup> [demeton-O-methyl sulfoxide, O,O-dimethyl S-2-(ethylsulfinylethyl) phosphorothioate]	Chemagro	m.p. = 73-74° "Pure"	1.00	Benzene
Malathion [S-(1,2-bis(ethoxycarbonyl)ethyl) O,O-dimethyl phosphorodithioate]	Cyanamid	b.p. = 106° at 0.01 mm 100%	1.00	Methanol
Meta-Systox-I <sup>a</sup> [methyl demeton, O,O-dimethyl S-(2-(ethylthio)ethyl) phosphorodithioate]	Chemagro	"Pure"	1.00	Methanol
Methyl parathion (O,O-dimethyl O- <i>p</i> -nitrophenyl phosphorothioate)	Shell	b.p. = 89° at 0.15 mm 99 + %	1.00	Methanol
Para-Oxon (O,O-diethyl O- <i>p</i> -nitrophenyl phosphate)	Cyanamid	Commercial	1.00	Methanol
Parathion (O,O-diethyl O- <i>p</i> -nitrophenyl phosphorothioate)	American Potash	98.5%	1.00	Methanol
Phosfon <sup>d</sup> (2,4-dichlorobenzyltributyl phosphonium chloride)	Virginia-Carolina Chemical	Commercial	1.00	Methanol

Compound	Dow	99 % "Pure"	Concentration	Solvent
Ruelene <sup>a</sup> (4-tert.-butyl-2-chlorophenyl methyl methylphosphoramidite)	Chemagro	—	1.00	Methanol
Systox <sup>a</sup> [demeton, mixture of O,O-diethyl O-2-(ethylthio)ethyl phosphorothioate and O,O-diethyl S-2-(ethylthio)ethyl phosphorothioate]	Victor	—	1.00	Methanol
Monoethyl acid orthophosphate	Aldrich Chem. Co.	—	1.00	Benzene
Chloromethylphosphonic acid	Aldrich Chem. Co.	—	1.00	Methanol
O,O-Diethyl chloromethylphosphonate	City Chemical Co.	—	1.00	Methanol
O,O-Dipropyl chloromethylphosphonate	Aldrich Chem. Co.	—	1.00	Methanol
O,O-Dibutyl chloromethylphosphonate	Aldrich Chem. Co.	—	1.00	Benzene
O,O-Diethyl cyanomethylphosphonate	Aldrich Chem. Co.	—	1.00	Benzene
O,O-Diethyl ethylphosphonate	Eastman	—	1.00	Methanol
O,O-Diethyl ethoxycarbonylmethylphosphonate	Eastman	—	1.00	Methanol
Methylphosphonic acid	Synthesized	—	1.00	Methanol
O-Methyl methylphosphonic acid	Synthesized	—	1.00	Methanol
Ethylphosphonic acid	Synthesized	—	1.00	Methanol
O-Ethyl ethylphosphonic acid	Synthesized	—	1.00	Methanol

<sup>a</sup> Chemagro Corporation trademark.

<sup>b</sup> Shell Chemical Company trademark.

<sup>c</sup> Hercules Powder Company trademark.

<sup>d</sup> Virginia-Carolina Chemical Company trademark.

<sup>e</sup> Dow Chemical Company trademark.

ture at the surface of the chromatoplate is 250°. The amount of spray reagent required will depend upon the area to be sprayed. In these studies, about 0.5 ml was sprayed on each microchromatoplate.

*Method I. Detection of phosphate pesticides and related compounds.* Spray the plate with the HI reagent, place the former on the hot plate adjusted so that the temperature on the surface of the plate is 250°, heat it for 15 min, and allow it to cool. Spray the plate with the ammonium molybdate reagent, wait 3 min, and spray it with the benzidine reagent. Phosphate and thiophosphate pesticides, and related phosphorus acids will give a blue spot against a white background. The colors are stable up to 8 h, longer if the plates are protected from light. Certain organophosphonates also give a blue spot, as noted below.

*Method II. Detection of phosphonate and phosphate pesticides and related compounds.* Spray the plate with the HI reagent, place it on the hot plate adjusted so that the temperature on the surface of the plate is 250°, and heat it for 15 min. Allow it to cool, spray it with the ammonium persulfate reagent, heat it again for 15 min, and allow it to cool. Spray the plate with ammonium molybdate reagent, wait 3 min, and spray it with the benzidine reagent. Phosphonate and phosphate pesticides, related thio compounds, and related phosphorus acids will give a blue spot against a white background.

*Differentiation among orthophosphate, organophosphates, and organophosphonates.* Orthophosphate will give a blue spot with both of the previous spray tests; however, it will also give a blue spot without using the HI reagent. Organophosphonates, with some exceptions, will not give a positive test in Method I. Consequently, different structures may often be identified on a plate by observing the response after spraying with ammonium molybdate plus benzidine without any pretreatment, after use of Method I, and after use of Method II.

## RESULTS AND DISCUSSION

Methods I and II were tested on the solutions of pesticides and other organophosphorus compounds listed in Table I. In these tests, 2, 5, and 10  $\mu\text{g}$  of each compound were spotted on a Silica Gel G microchromatoplate, and the plate was sprayed with the appropriate reagents. All of the phosphate pesticides and the monoethyl acid orthophosphate were detected at the 2- $\mu\text{g}$  level with Method I. In addition 2  $\mu\text{g}$  each of chloromethylphosphonic acid, O,O-diethyl chloromethylphosphonate, O,O-dipropyl chloromethylphosphonate, and O,O-dibutyl chloromethylphosphonate were detected with Method I. All of the compounds were detected at the 2- $\mu\text{g}$  level with Method II except EPN and Phosfon. EPN was detected at the 5- $\mu\text{g}$  level, while Phosfon produced a yellow spot at the 2- $\mu\text{g}$  level. The blue spots produced by EPN intensified with time. In Method I, brown rings formed at the periphery of the spot after heating, and prior to spraying, with the ammonium molybdate solution. The intensity of the rings appeared to be affected by the functional group joined through oxygen to the central phosphorus atom. The most intense rings were produced by compounds containing large functional groups, especially phenyl and substituted phenyl groups.

### *Sensitivity*

The sensitivity of the spray tests for all of the compounds was not determined, principally because the sensitivity obtained with any chromogenic agent used for thin-layer chromatography (TLC) depends upon the technique used to apply the agent. Except as noted, these spray tests readily detected 2  $\mu\text{g}$  of pesticide or acid when applied by the techniques described in these studies. The intensities of the spots obtained at these levels indicate that at least 1  $\mu\text{g}$  of most phosphate pesticides can be detected.

### *Selection of spray reagents*

A reagent containing hydriodic acid, acetic acid, and phenol is widely used for the determination of the alkoxy function<sup>16</sup>; it has also proved effective for the hydrolysis of a variety of organophosphorus compounds<sup>17</sup>. Because the acetic acid and phenol are believed to function primarily as solvents for the solid ethers produced<sup>16</sup>, reagents not containing one or both of them were tested as spray reagents. Satisfactory results were obtained only if the spray reagent contained acetic as well as hydriodic acid, while phenol could be omitted without affecting the tests. Concentrations of both the acetic and hydriodic acids in the reagent were not critical within wide limits.

The HI reagent hydrolyzed not only all organic phosphates tested, but also some organic phosphonates. All of these compounds were substituted on the carbon atom attached to the central phosphorus atom. These experiments indicated that the cleavage of the P-C bond is accelerated by certain substituents on this carbon atom.

Ammonium persulfate has been used for the oxidation of methylphosphonic acid in acid solution to orthophosphate<sup>18</sup>, and of tetramethylphosphonium chloride and other organic phosphorus compounds<sup>19</sup> to orthophosphate. However, O-alkyl alkylphosphonates could not be converted to orthophosphate on TLC plates with acid solutions of ammonium persulfate. Apparently persulfate will cleave the P-C bond but not the P-O-C bond under these conditions. Spraying with ammonium persulfate followed by HI produced only faint blue spots. The concentration of ammonium persulfate and the acidity of the reagent are not critical within wide limits.

The method and time of heating the plates following spraying with the HI and ammonium persulfate reagents were studied extensively. Heating under infrared heat lamps was not satisfactory because the required temperatures could not be attained on the surface of the plate. Also, the temperature attainable at a fixed distance from the lamp varied with the age of the lamp; at a distance of 3 in. a 75° difference was noted between a new and an old lamp. Heating at temperatures above 250° occasionally cracked the plates and did not noticeably increase the intensity of the spots, while heating at temperatures below 250° after spraying with ammonium persulfate reagent produced less intense spots. Heating for 15 min was required to remove residues from the plate which otherwise interfered with the development of the molybdenum blue color.

Many combinations of reagents have been developed for producing the molybdenum blue color with orthophosphate. The sensitivity and reliability of four popular combinations, ammonium molybdate and benzidine<sup>20</sup>, ammonium molybdate and Elon (*p*-methylaminophenol sulfate)<sup>21</sup>, ammonium molybdate and 1,2,4-triaminonaphtholsulfonic acid (ANS)<sup>21</sup>, and ammonium molybdate and hydroquinone<sup>21</sup>, were

determined by spotting 1  $\mu$ l of a solution containing 1 mg/ml of phosphorus, as sodium dihydrogen phosphate, on a Silica Gel G microchromatoplate and spraying it consecutively with the two reagents. The ammonium molybdate solutions contained 2 % (w/v) ammonium molybdate in 1 M HClO<sub>4</sub> or 1 M HCl; the benzidine solution contained 0.05 % (w/v) benzidine, 22.5 % (w/v) sodium acetate trihydrate and 10 % (w/v) glacial acetic acid in water; the Elon solution contained 0.1 % (w/v) Elon, 2 % (w/v) sodium bisulfite, and 0.5 % (w/v) sodium sulfite in water; the ANS solution contained 0.1 % (w/v) 1,2,4-triaminonaphtholsulfonic acid, 2 % (w/v) sodium bisulfite, and 0.1 % (w/v) sodium sulfite in water; and the hydroquinone solution contained 0.1 % (w/v) hydroquinone. The greatest sensitivity was obtained with the ammonium molybdate-benzidine combination. Both solutions of ammonium molybdate gave satisfactory results, but the HCl solution was more stable. The benzidine solution probably gives the greatest sensitivity because of the formation of benzidine blue as well as molybdenum blue. The molybdate-benzidine reaction produces the most intense blue color in slightly acid solution; the sodium acetate-acetic acid buffer provides the correct pH for the reaction and eliminates interferences produced by residual acid or base on the plate. No blue spot is produced if the sodium acetate is omitted from the benzidine solution. The concentration of reagents in the ammonium molybdate and benzidine solutions is not critical within wide limits.

#### *Effect of solid phase*

Both spray tests are equally satisfactory on either Silica Gel G or Kieselguhr G. If Silica Gel H (containing no binder) is used as a solid phase for thin-layer chromatography, it tends to flake off the plate following spraying with ammonium persulfate and heating. This flaking can be minimized by using a low-velocity sprayer.

#### *Applications of spray tests*

These spray tests should be useful for detecting a wide variety of phosphate and thiophosphate pesticides, and their decomposition products on thin-layer chromatograms. Properly used, the spray tests may also provide information about the bonding in the phosphorus compound.

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#### SUMMARY

Organophosphates, organophosphonates, organophosphorus acids, and related thio compounds can be detected on thin-layer chromatoplates by two new spray tests. In one, plates are sprayed with hydriodic acid solution, heated at 250°, and sprayed with ammonium molybdate solution followed by buffered benzidine solution. In the other, plates are sprayed with hydriodic acid solution, heated, sprayed with ammonium persulfate solution, heated again, and sprayed with ammonium molybdate solution followed by buffered benzidine solution. Hydriodic acid cleaves P-O-C bonds, while ammonium persulfate cleaves P-C bonds, and the resulting orthophosphate is detected by formation of molybdenum and benzidine blue. All of the

organophosphates and organophosphoric acid plus some of the organophosphonic acids gave a positive test after use of the HI reagent but not the ammonium persulfate reagent, while all of the compounds that were tested except a phosphonium compound gave positive tests after use of both the HI and ammonium persulfate reagents. The tests can be used to distinguish among orthophosphates, organophosphates, and some organophosphonates. As little as 2  $\mu\text{g}$  of many pesticides and organophosphorus acids can be detected, and the ultimate lower limit of detection of the tests is less than 1  $\mu\text{g}$ .

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