notes on methodology

A simple, specific spray for the detection of phospholipids on thin-layer chromatograms

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A SPRAY reagent specific for phosphate esters on thin-layer plates is particularly useful in the identification of phospholipids. Ammonium molybdate–perchloric acid spray (1), which has been widely used for the detection of phospholipids on paper, is not satisfactory when used on silica gel plates. Color development is slow at room temperature and long development or heating the plates leads to non-specific reactions with most lipids. In fact, a modification of this reagent is used as a general non-specific spray for lipids (2). The phosphate spray of Jatzkewitz and Mehl (3) is reported to be specific but its use requires several steps and considerable manipulation. A modification of the molybdenum blue reagent of Zinzadze (4) is described here, which, when used as a spray, gives an instantaneous, specific reaction with phospholipids on silica gel or alumina plates. The mechanism of the reaction is not known at present.

Source and Preparation of Lipids. Ox brain and liver lipids were extracted with chloroform–methanol 2:1 (v/v) (5). Egg yolk phospholipids were extracted with chloroform–methanol 1:1 after first extracting with acetone (6). Egg and liver phosphatidyl ethanolamine and phosphatidyl choline, and liver phosphatidyl inositol, were prepared by chromatography on silicic acid (7, 8). Brain cerebroside, sphingomyelin, and cerebroside sulfate were prepared by a combination of chromatographic procedures on silicic acid and alumina to be described elsewhere. Brain phosphatidyl inositol diphosphate was prepared as described by Dittmer and Dawson (9). Ceramide was prepared by hydrolysis of brain sphingomyelin (10) and purified by chromatography on silicic acid. Sphingosine was prepared by hydrolysis of crude brain sphingolipids (11). Stearic, palmitic, and myristic acids and their methyl esters, and behenyl alcohol, tripalmitin, tristearin, cholesterol, and cardiolipin[1] were all commercial preparations.

Thin-layer plates were prepared from Silica Gel G and Aluminum Oxide G (25 g of the adsorbent to 50 ml of water) as recommended by the distributor.[2] Plates were air-dried, heated at 100° for 1 hr, and stored in a desiccator over anhydrous $CaSO_4$. The most useful solvents were found to be chloroform–methanol 4:1 (v/v) with 2% water and 2% pyridine added for silica gel plates, and chloroform–methanol 3:2 (v/v) with 5% water for the alumina plates.

Molybdenum blue reagent was prepared as described by Zinzadze (4) except that titration with $KMnO_4$ to determine the final concentration is not necessary for this application. Reagent grade chemicals were used throughout.

Solution I. To 1 liter of 25 N H_2SO_4 40.11 g of MoO_3 is added and the mixture is boiled gently until the MoO_3 is dissolved.

Solution II. To 500 ml of Solution I 1.78 g of powdered molybdenum[3] is added and the mixture is boiled gently for 15 min. The solution is cooled and decanted from any residue that may be present.

Molybdenum Spray. Equal volumes of Solutions I and II are mixed and the combined solution is mixed with two volumes of water. The final solution is greenish yellow in color. If too little water is used it will be blue; if too much, yellow. The spray is stable for months. Plates are sprayed lightly until the adsorbent is uniformly damp. Compounds containing phosphate ester show up immediately as blue spots on a white or light blue-grey background. The intensity of the color increases on standing. After several hours, the background darkens to a deep blue and the spots are obscured. Plates cannot be kept as a permanent record.

Ninhydrin Spray. A 0.2% solution of ninhydrin[4] in acetone is diluted with an equal volume of water immediately before using. After spraying, the plates are allowed to stand at room temperature for 2 or 3 hr or are heated for 5 min at 100°.

Rhodamine 6G Spray. A 0.005% solution of Rhodamine 6G[5] in water is sprayed on the plate until it is uniformly damp. The plate is viewed under a UV lamp while still wet.

Because maximum information can be obtained from a chromatogram by using several sprays in succession, the phosphate spray was tried in several combinations before and after the use of both Rhodamine 6G (as a universal lipid spray) and ninhydrin (as a spray specific for amino

[1] The Sylvana Co., Millburn, N. J.

[2] The adsorbents are products of E. Merck, A. G., Darmstadt, Germany, and are distributed by C. A. Brinkmann and Co., Great Neck, N.Y.

[3] Molybdic anhydride and molybdenum metal are products of Fisher Scientific Co., St. Louis, Mo.

[4] Mann Research Laboratory, Inc., New York City.

[5] National Aniline Division, Allied Chemical Corp., New York City.

groups). The most satisfactory procedure was to spray the plate first with Rhodamine 6G and stipple around the spots with a sharp pencil. After the plate is completely dry, it is sprayed with ninhydrin and the spots which give a positive reaction are noted. Finally, the plate is treated with molybdenum spray. When the molybdenum spray is used after Rhodamine 6G or ninhydrin treatment, the rate at which the background darkens is increased and the spots may be obscured in as short a time as half an hour. Because the reaction with phosphate esters is instantaneous, this presents no particular difficulty.

If a tracing is to be made of the plate, this is done before spraying for phosphate because the acid in the phosphate spray destroys tracing paper. A satisfactory permanent record can be obtained by photographing the plate with a Polaroid Land camera under UV light after spraying with Rhodamine 6G (12). Reactivity with ninhydrin and molybdenum sprays can be noted subsequently on the print.

The *sensitivity* of the phosphate spray was determined on serial dilutions of egg lecithin and phosphatidyl ethanolamine. As little as 0.005 μmole of both phosphatidyl ethanolamine and phosphatidyl choline could be detected when the molybdenum spray was used alone. The sensitivity under these conditions is equivalent to that obtained with Rhodamine 6G with both phospholipids and is from two to three times as sensitive as ninhydrin with phosphatidyl ethanolamine. When the molybdenum spray is used after Rhodamine 6G or ninhydrin spray or both, its sensitivity decreases to approximately 0.01μmole.

Specificity. A wide range of compounds including phosphatidic acid, cardiolipin, sphingomyelin, phosphatidyl ethanolamine, -serine, -choline, -inositol, and -inositol diphosphate were all found to give positive reactions with the molybdenum spray. Fatty acids, fatty acid methyl esters, triglycerides, long-chain alcohols, cholesterol, ceramide, sphingosine, cerebroside, and cerebroside sulfate do not give a positive reaction.

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