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Note

Modified Jungnickel's reagent for detecting phospholipids and other phosphorus compounds on thin-layer chromatograms

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At present, there are some useful reagents[1–6] for detecting phospholipids on thin-layer chromatograms, but none of them gives colour reactions with phosphorus-containing polar products of phospholipid degradation. These products are usually rendered visible by the method of Hanes and Isherwood[7], but their original or modified method has the disadvantages of low reagent stability, poor sensitivity and small contrast between spots and background[8].

Some different methods for the detection of organophosphorus compounds have been suggested during the last decade[8–12]. They are based on the same general principle as the Hanes and Isherwood procedure, namely the hydrolysis or digestion of the compound to give an inorganic phosphate, followed by a colour reaction of the latter substance. These methods are used mainly in pesticide chemistry, but not in phospholipid biochemistry.

We have elaborated a procedure, based on these methods, for detecting very small amounts of phospholipids and the products of their hydrolysis on micro-thin-layer chromatograms.

MATERIALS AND METHODS

Crystal violet, malachite green, sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), potassium dihydrogen orthophosphate (KH_2PO_4), ammonium peroxydisulphate ($(NH_4)_2S_2O_8$) and hydrochloric, sulphuric and perchloric acids were commercial preparations used without additional purification.

Lipid extracts and egg-yolk phosphatidylcholine were obtained as described previously[6]. The micro-scale thin-layer chromatographic (TLC) technique has been described earlier[13].

Preparation of spray reagents

The spray reagents were prepared from a stock solution of basic dyes (10 mg/ml) in 2 *N* hydrochloric acid and sodium molybdate (50 mg/ml) in 2 *N* hydrochloric acid. To 1–4 ml of dye solution, a calculated amount of hydrochloric acid and 0.5–2.0 ml of the molybdate solution were added while mixing, and the volume was adjusted to 10 ml with water. After half an hour, the mixture was filtered through filter-paper.

Investigation of digestive reagents and sprays

Amounts of 0.01, 0.05, 0.1 and 0.5 μg of phosphorus as a KH_2PO_4 solution and 0.1, 0.5 and 1.0 μg of phosphatidylcholine dissolved in chloroform were spotted on to micro-TLC plates in areas of 5-mm diameter. The plates were sprayed with a digestion reagent and placed on an electric heater with a surface temperature of about 250°. The plates were then cooled and sprayed with a detection reagent. In some of the experiments, the inorganic phosphate spots were sprayed with the detection reagent only.

Recommended procedures

Preparation of malachite green spray reagent. Add a solution of $Na_2MoO_4 \cdot 2H_2O$ (5 g) in 2 *N* hydrochloric acid (150 ml) to a solution of malachite green (1 g) in 2 *N* hydrochloric acid (100 ml); mix and dilute to 500 ml with water. After 1–2 h, filter the mixture through filter-paper. Keep the reagent in a dark bottle at room temperature; it remains stable for several months. The sediment that forms during storage does not influence the quality of the reagent: filter the spray before use.

Detection of phosphorus compounds with dye spray. Completely remove organic solvents from the developed chromatoplates: spray the plates thoroughly with 57–72% perchloric acid and place them on hot-plates with a surface temperature of 250–300°. After the disappearance of dark spots and evaporation of the perchloric acid as a white vapour, remove the TLC plates from the hot-plate and, after cooling, spray them thoroughly with the malachite green reagent so as to obtain a uniform orange background. After a few minutes, bright green spots of phosphorus compounds will appear on this background.

Consecutive detection of phospholipids with different sprays. Spray the chromatogram with ninhydrin or Dragendorf reagent. After spots have appeared, spray the plate with molybdate reagent A[6]. Then heat it on a hot-plate, detecting all of the spots with a sulphuric acid solution of the same reagent. Finally, perform TLC in accordance with the above procedure for the detection of phosphorus compounds. Record the spots on a clean glass plate after every detection.

RESULTS AND DISCUSSION

Both stages of the Hanes and Isherwood method (decomposition of an organophosphorus compound to inorganic phosphate and detection of the latter) need improvement. Barney[8] suggested hydrolyzing the compounds on plates with hydriodic acid in glacial acetic acid at 250° or digesting them with ammonium peroxydisulphate at the same temperature. Washüttl and Bancher[10] conducted the decomposition with hydroperoxide at 110°. Stenersen[11] sprayed the TLC plates with a solution of ammonium molybdate in a hydrochloric plus perchloric acid and heated the gel side of the plate directly with the flame of a burner, or placed the plate in an oven for 1 h at 200°. Askew *et al.*[12] modified the Barney procedure by lowering the temperature to 180° and covering the TLC plates with a glass plate during heating. In order to detect the inorganic phosphate obtained in TLC, phosphomolybdenum blue[8,10,12] or basic dyes[9,11] have been used.

We began our work by investigating the optimal conditions for the detection of inorganic phosphate on TLC plates and decided to use basic dye sprays.

The molar absorptivities of phosphate with crystal violet in aqueous solution and with malachite green are $8.1 \cdot 10^4$ and $7.3 \cdot 10^4$, respectively[14]. Phosphomolybdenum blue has a molar absorptivity of about $2.7 \cdot 10^4$ (ref. 14) and undegraded phospholipids give a value of $7.5 \cdot 10^3$ (ref. 15) in the solution with Zinzadze reagents. One would therefore expect that basic dyes would give the most sensitive spray reagents.

We first investigated the influence of basic dyes, molybdate and acid concentrations on spray quality. Jungnickel[9] took 4.5 mg of dye, about 5 mg of molybdenum as ammonium molybdate and 1.1 mequiv. of hydrochloric acid per millilitre of his reagent. The dyes were dissolved in water. We found it more satisfactory to use hydrochloric acid and then to mix the solution with a solution of molybdate in hydrochloric acid. Although crystal violet gives a greater absorbance than malachite green with phosphate[14], and has been used for phosphate sprays[9,11], it gave a voluminous sediment with molybdate and the resulting spray after filtration was less sensitive than that with malachite green, and we therefore decided to use the latter. Subsequent investigations were carried out with malachite green.

When we detected inorganic phosphate on the plates, the best results (bright green spots on a light orange background) were obtained with a reagent containing 2 mg of the dye, 10 mg of molybdenum and 2 mequiv. of hydrochloric acid per millilitre. At acid concentrations below 1 *N*, the background was green; at concentrations of 2.5 *N* and above, spots were absent and the background was dark orange.

In the first experiments initiating digestion, the plates with inorganic phosphate spots were sprayed with sulphuric or perchloric acid and subsequently heated; these runs showed that the reagent did not detect phosphate, the plates being uniformly dark orange. This effect is probably due to the acid binding with silica gel as an ion exchanger. We therefore tested a new series of reagents. Good results were obtained with sprays containing 2–4 mg of the dye, 3–8 mg of molybdenum and an 0.8–1.0 mequiv. of hydrochloric acid per millilitre. The reagent whose composition is given under *Recommended procedures* showed clearly 0.01 μg of phosphorus in a 5-mm diameter spot.

Finally, we established the optimal conditions for the decomposition of organophosphorus compounds on micro-TLC plates. Phosphatidylcholine was taken as a model substance. Heating of phosphatidylcholine spots with sulphuric acid, hydroperoxide and ammonium peroxydisulphate did not give the desired results and black spots usually remained on the plates, which interfered with the phosphate colouring and decreased the sensitivity of the reagent. We then tested combustion with perchloric acid alone or with small amounts of molybdate, which accelerated the degradation of organic phosphate[16]. On a hot-plate with a surface temperature of 250–300°, complete destruction of phospholipids occurred within 2–3 min with no loss of phosphate. As a result, we developed a method for the detection of organophosphorus compounds (see *Recommended procedures*). The basic dye reagent proved to be as sensitive as sulphuric acid. It was possible to detect as little as 0.2–0.3 μg of the phosphorus compound on micro-TLC plates.

The method is convenient for detecting not only the products of phospholipid hydrolysis, but also the phospholipids themselves. It allows minor components to be detected and phospholipid spots to be distinguished among the major lipid spots detected previously on TLC plates with sulphuric acid.

The method gives very useful information for investigating complex lipid

mixtures, especially when used as the last step of successive TLC stages with different sprays (see *Recommended procedures*).

REFERENCES

1 J. C. Dittmer and R. L. Lester, *J. Lipid Res.*, 5 (1964) 126.
2 V. E. Vaskovsky and E. Y. Kostetsky, *J. Lipid Res.*, 9 (1968) 396.
3 S. K. Goswami and C. F. Frey, *J. Lipid Res.*, 12 (1971) 509.
4 V. E. Vaskovsky and V. I. Svetashev, *J. Chromatogr.*, 65 (1972) 451.
5 V. Giridhar, F. R. Dalal and S. Winsten, *Clin. Chem.*, 20 (1974) 513.
6 V. E. Vaskovsky, E. Y. Kostetsky and I. M. Vasendin, *J. Chromatogr.*, 114 (1975) 129.
7 C. S. Hanes and F. A. Isherwood, *Nature (London)*, 164 (1949) 1107.
8 J. E. Barney, II, *J. Chromatogr.*, 20 (1965) 334.
9 F. Jungnickel, *J. Chromatogr.*, 31 (1967) 617.
10 J. Washüttl and E. Bancher, *Mikrochim. Acta*, (1967) 395.
11 J. Stenersen, *J. Chromatogr.*, 38 (1968) 538.
12 J. Askew, J. H. Ruzicka and B. B. Wheals, *Analyst (London)*, 94 (1969) 275.
13 V. I. Svetashev and V. E. Vaskovsky, *J. Chromatogr.*, 67 (1972) 376.
14 A. K. Babko, Y. F. Shkaravsky and V. I. Kulik, *Zh. Anal. Khim.*, 21 (1966) 196.
15 R. K. Raheja, C. Kaur, A. Singh and I. S. Bhatia, *J. Lipid Res.*, 14 (1973) 695.
16 R. M. C. Dawson, *Biochem. J.*, 75 (1960) 45.