CHROM. 5790

Phospholipid spray reagents

A reagent specific for the detection of phospholipids on chromatograms is of the utmost importance to the study of phospholipids. Among such reagents, the most widely used today is the one described by DITTMER AND LESTER[1]. Actually, this is a modification of ZINZADZE's reagent[2] originally used in the determination of phosphorus. We have previously reported an even simpler method for preparing a phospholipid spray[3], this method being based on the use of the LUSENA-CONDE-PRAT reagent[4]. On the other hand, HAHN AND LUCKHAUS[5] described a further modification for obtaining a reduced molybdate reagent to determine phosphorus.

We now report a new phospholipid spray reagent based on this latter modification, but essentially simpler to use and less corrosive.

Experimental

Materials and methods. Lecithin was isolated from the lipids of a hen egg by means of chromatography on alumina and silica gel. The sodium molybdate, hydrazine hydrochloride, hydrochloric acid and sulphuric acid were all chemically pure reagents. The following stock solutions were used to check the properties of the reagent prepared: (1) sodium molybdate (20 g) in 0.5 l of 2 *N* HCl and (2) hydrazine hydrochloride (1.93 g) in 0.5 l of water.

From 0 to 18 ml of the latter were added to 25 ml of the former solution, and the volume of the mixture was adjusted to 50 ml with water. After subsequent heating for 5 min on a boiling-water bath and cooling, the mixture was diluted with water and acids to 100 ml. The reagent obtained was sprayed on a plate on to which 1, 2, 5 and 10 μg of lecithin were spotted in 5 mm diameter areas. Following this, we observed the rate at which colouring occurred and its stability and background alteration. At the same time, we varied the molybdate and hydrazine concentrations (reducing from Mo (VI) to Mo(V)), and the quantity and quality of the acids used.

Thin-layer chromatography. Silica Gel KCK (150–200 mesh) with gypsum was used for thin-layer chromatography (TLC). A chloroform(C)–methanol(M)–water (65:25:4) mixture[6] was the solvent system used for one-dimensional chromatography. The following solvents were used for two-dimensional chromatography: (1) C–M–28% aqueous ammonia (65:35:5) and (2) C–acetone–M–acetic acid–water (50:20:10:10:5) (ref. 7).

Investigation of the reagent mechanism. Pure egg lecithin was spotted on to the plate starting line in the form of bands. Some of the bands were sprayed with the phospholipid reagent, the rest being detected with a solution of iodine in methanol. After the zones were dry and iodine had evaporated, one-dimensional development was carried out. Following secondary detection with the phospholipid spray or charring with sulphuric acid, phosphorus was determined in the spots[8].

Recommended procedure for preparing the reagent. Sodium molybdate (10 g) was dissolved in 100 ml of 3–4 *N* HCl and hydrazine hydrochloride (1.0 g) was dissolved in 100 ml of water. Both solutions were then mixed and heated on a boiling-water bath for 5 min. After cooling, the volume was adjusted to 1 l with water and the reagent was then ready for use. It proved to be stable for at least several months.

Results and discussion

HAHN AND LUCKHAUS' reagent[5] has been used previously as a phospholipid reagent in paper chromatography[9,10] and TLC[11]. In their work, LONG *et al.*[11] and BEISS[10] used this reagent unmodified with a 100-ml content of concentrated sulphuric acid per litre of ready-for-use reagent. Later, BEISS[9] described a reagent containing 250 ml of concentrated acid per *ca.* 950 ml of the reagent. Incidentally, KATES[12] cited this procedure in his review on the paper chromatography of lipids.

When conducting initial tests on HAHN AND LUCKHAUS' preparation[5] as a phospholipid spray, we noticed that the reagent with a lower acid content was not always effective. This may be due to two causes: insufficient acid concentration or greater heat evolution with increased acid content. Indeed, it turned out that a reagent with a lower acid content gave good results when heated for several minutes on a boiling-water bath, before or after having been diluted with water. A study of the data[13,14] on conditions for reducing Mo(VI) to Mo(V) showed that, in 0.3–2.0 *N* HCl at 100°, reduction with hydrazine occurs in 2–5 min. With acid concentration lower than 0.3 *N*, molybdenum blue forms, whereas with hydrochloric acid concentrations above 2 *N*, reduction is delayed. Reduction is, to a great extent, also inhibited by sulphuric acid; even a large excess of hydrazine sulphate does not reduce more than 80 % of the molybdenum[14]. Hydrazine hydrochloride has another advantage over the sulphate, namely better solubility (*ca.* one hundred-fold). It was precisely on account of this that HAHN AND LUCKHAUS[5] preferred sodium molybdate to ammonium molybdate for their reagent.

We reduced Mo(VI) with hydrazine hydrochloride in *N* HCl for 5 min, boiling the reaction mixture on a water bath. We then selected a reagent with optimal composition, varying the molybdate, hydrazine and acid concentrations.

As with our first reagent[3], sufficiently good results were obtained with a wide range of molybdenum concentrations, extent of reduction (excluding 0 % and 100 %) and acid concentrations. The method is essentially simpler than the technique of DITTMER AND LESTER[1] and our previous techniques[3].

Another advantage of the new reagent lies in its significantly lower content of acid compared with other phospholipid reagents[1,3,9,10].

Phospholipids produce blue spots on a white background immediately after spraying and 1–2 h later a blue background develops. Spraying with 10 % sulphuric acid in methanol is a means of avoiding this.

Another problem of paramount importance is the action mechanism of the reagent. DITTMER AND LESTER[1] maintain that this mechanism is unknown. However, on studying our previous reagent[3], we found that, on butanol extraction, the complex resulting from the combination of the phospholipid with the reagent breaks down to give an undegraded phospholipid (not less than 70 %). On testing the destructiveness of the present reagent by chromatography of lecithin after treatment by the spray on the starting line, we found that about 75 % of the phospholipid remains undestroyed. *Ca.* 15 % of the phosphorus was detected in the blue spot situated in the C–M–water (65:25:4) system below lecithin. Thus, it was shown that the coloured product is essentially an unstable complex of the phospholipid and the reagent mixture of reduced and unreduced molybdate. Recently, GALANOS[15] described the formation of a complex involving unreduced molybdate and phospholipids.

At present, reagents for phopholipid detection based on Mo(VI)–Mo(V) (fre-

quently called ZINZADZE's reagents, irrespective of the method used to reduce the molybdenum) have supplanted those reagents which are based on preliminary hydrolysis of the lipid on a plate with subsequent identification of inorganic phosphorus[16,17].

ZINZADZE's reagent[2] was first used by KANNGISSER[18] to detect nucleic acids by paper electrophoresis.

Later, KANNGISSER[19] found that lecithin colours much more intensely than nucleic acids, and then purposely used ZINZADZE's reagent to detect phospholipid zones on the electropherograms[20]. Still later, BEISS AND AMBRUSTER[21] used two modifications of KANNGISSER's method to detect phospholipids on paper chromatograms. According to information available in the literature, a reagent based on ZINZADZE's preparation has also been used by SOUČEK *et al.*[22].

Even when unmodified, HAHN AND LUCKHAUS' reagent[5] is simpler to prepare and of no worse quality than ours[3] or the reagent of DITTMER AND LESTER[1]. Yet it is still not very popular among biochemists for phospholipid detection. Apparently this is because publications on the use of the reagent as a phospholipid spray were originally made, not in a special communication, but in extensive research papers[9,10] which were lost to specialists.

Institute of Biologically Active Substances,
F. E. Research Centre, Academy of
Sciences of the U.S.S.R., Vladivostok-22 (U.S.S.R.)

V. E. VASKOVSKY
V. I. SVETASHEV

1 J. C. DITTMER AND R. L. LESTER, *J. Lipid Res.*, 5 (1964) 126.
2 C. ZINZADZE, *Ind. Eng. Chem.*, 7 (1935) 227.
3 V. E. VASKOVSKY AND E. Y. KOSTETSKY, *J. Lipid Res.*, 9 (1968) 396.
4 F. LUSENA-CONDE AND L. PRAT, *Anal. Chim. Acta*, 16 (1957) 473.
5 F. L. HAHN AND R. LUCKHAUS, *Z. Anal. Chem.*, 149 (1956) 172.
6 H. WAGNER, L. HÖRHAMMER AND P. WOLFF, *Biochem. J.*, 334 (1961) 175.
7 G. ROUSER, G. KRITCHEVSKY AND A. YAMAMOTO, *Lipid Chromatogr. Anal.*, 1 (1967) 145.
8 G. ROUSER, S. FLEISHER AND A. YAMAMOTO, *Lipids*, 5 (1970) 494.
9 U. BEISS, *J. Chromatogr.*, 13 (1964) 104.
10 U. BEISS, *Moderne Methoden der Pflanzenanalyse*, Vol. 6, 1963, p. 52.
11 C. LONG, R. ODAVIC AND E. G. SARGENT, *Biochem. J.*, 102 (1967) 216.
12 M. KATES, *Lipid Chromatogr. Anal.*, 1 (1967) 1.
13 A. I. BUSEV, *Zh. Anal. Khim.*, 14 (1959) 445.
14 G. G. RAP AND M. SURYANARAYA, *Z. Anal. Chem.*, 168 (1957) 177.
15 D. S. GALANOS, *Lipids*, 5 (1970) 573.
16 M. A. HACK AND V. G. FERRANS, *Z. Physiol. Chem.*, 315 (1959) 157.
17 H. JATZKEWITZ AND E. MEHL, *Z. Physiol. Chem.*, 320 (1960) 251.
18 W. KANNGISSER, *Naturwissenschaften*, 38 (1951) 503.
19 W. KANNGISSER, *Z. Physiol. Chem.*, 291 (1952) 247.
20 W. KANNGISSER, *Biochem. Z.*, 325 (1953) 12.
21 U. BEISS AND O. AMBRUSTER, *Z. Naturforsch.*, 138 (1958) 79.
22 A. SOUČEK, Č. MICHALEC AND A. SOUČKOVÁ, *Biochim. Biophys. Acta*, 144 (1967) 180.

Received September 7th, 1971