Antioxidants for the (thin-layer) chromatography of lipids

The danger of degradation by autoxidation is ever present when lipids are manipulated in the laboratory and particularly when they are dispersed on surface and exposed to air as in chromatography. An unstable material such as egg phosphatidylethanolamine, for example, has been observed to absorb the equivalent of one mole of oxygen per mole of phospholipid in an hour at 37° when spread on glass[1], indicating the danger of significant change even in a few minutes, and the rate of oxidation is greatly increased on exposure to light. As PRIVETT *et al.*[2] have pointed out the best method for safe storage of readily autoxidisable lipids is at low temperatures in dilute solutions in solvents as air-free as practicable, and the period of greatest danger, for example in thin-layer chromatography, is when the solvent has been removed from the developed plate. While a descending technique, with elution without drying, does give considerable protection[2], the speed and simplicity which are two of the thin-layer method's greatest assets are thereby lost, and the alternative of development in an inert gas atmosphere, when coupled with the necessity for presaturating with solvent, is also rather troublesome for routine use.

We have preferred to retard chemical change by developing the plates in air at 0°, in the absence of light, and to add an antioxidant to the developing solvent for additional protection. Under these conditions oxidative degradation seems to be reduced to negligible proportions.

Experimental

Material. Phosphatidylethanolamine, prepared from egg yolk lipid[1] and freed from nitrogenous impurities by passage through a cellulose column[3], was held in solution in chloroform containing 5 mg % BHT (butylated hydroxy toluene; 2,6-di-*tert.*-butyl-4-methylphenol) under carbon dioxide at —80° until required. The presence of an antioxidant was considered necessary to protect the lipid during storage, application to the plate and drying off of the solvent. Since BHT and BHQ (butyl hydroquinone; 1,4-dihydroxy-2-*tert.*-butyl benzene) both move with the solvent front the very small amount of either of these substances applied with the phospholipid sample would be washed out immediately the unstabilized solvent front reached it.

Chromatography. 20 × 20 cm plates of 300 μ activated silica gel H were divided into six lanes, the two outermost (1.25 cm wide) receiving 2 μl spots and the four inside lanes (3.75 cm wide) 25 μl streaks of sample solution (16 μg P/25 μl). Development was in a constant temperature room at 0° with a solvent of BOWYER AND HOWARD[4]. When the solvent front had moved 17 cm (in about 80 min) the outside lanes were sprayed with rhodamine–fluoresceine reagent[4] and the plate dried for 30 min in a stream of warm nitrogen. Runs were made under the conditions listed in Table I.

After disclosing the marker spots under the U.V. lamp the main part of the plate was scored and the silica carrying the phospholipid removed into centrifuge tubes (still at 0°) for extraction (in the laboratory) first with 2 × 5 ml portions of development solvent and then with 3 ml and 2 ml methanol.

Measurement of the degree of oxidation. The solvent was removed by evaporation under nitrogen and the residue taken up in 3 ml spectral grade cyclohexane for spectrophotometric examination, with measurement of extinction in the conjugated diene

TABLE I

EFFECT OF ANTIOXIDANTS ON THE AUTOXIDATION OF PHOSPHATIDYLETHANOLAMINE DURING THIN-LAYER CHROMATOGRAPHY IN AIR AT 0°

Conditions		*Increase in extinction* at 232 mμ*	
		Expt. 1	*Expt. 2*
A	Dried with warm N_2 and eluted	0.43	0.27
B	As A, but with 5 mg% BHT in solvent	0.24	0.14
C	As A, but with 10 mg% BHT in solvent	0.08	0.01
D	As A, but with 5 mg% BHQ in solvent	0.21	0.16
E	As A, but with 10 mg% BHQ in solvent	0.06	0.00
F	As A, but dried 2 h in air and sunlight**	1.48	—
G	As F but with 10 mg BHT in solvent	0.90	—

* Initial value (H) 0.12.
** Through glass, early February, 52° N latitude.

region near 232 mμ, a "blank" being simultaneously run on an extract prepared from a phospholipid-free part of the plate. Finally, the phosphorus content of the eluted sample was determined after removal of the cyclohexane solvent, recovery being approximately 97 % of the phosphorus applied.

Results

The sample of egg phosphatidylethanolamine used showed a small absorption at 232 mμ (Table I), though no peak (Fig. 1). The molar extinction of the hydroperoxides formed from linoleate in the early stages of autoxidation varies to some extent with conditions, but is known to be of the order of 28,000 (ref. 5). On this basis the fatty acid residues in the phospholipid preparation used cannot have been more than about 1 % oxidised.

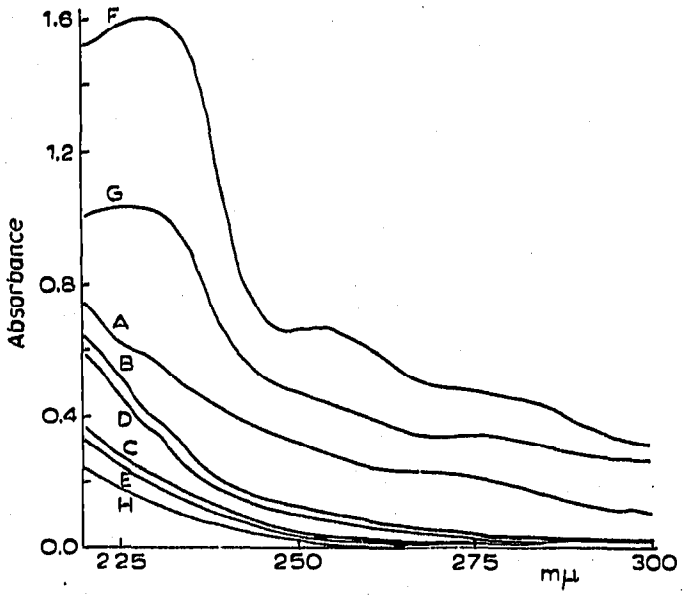

Fig. 1. Absorption of phosphatidylethanolamine in cyclohexane solution after thin-layer chromatography in presence and absence of antioxidants. For conditions see Table I.

During chromatography at 0°, without antioxidant, and recovery from the plate by the usual method the level of oxidation increased several fold (Table I, A) but was still quite small. However, with even moderate exposure to air and daylight the change, at about 16 % of the fatty acids peroxidised (Table I, F), became much more serious.

The antioxidants afforded useful protection against autoxidation in normal handling though, as expected, they were less effective against direct sunlight. In previous work on the inhibition of the autoxidation of unsaturated fatty acid esters[6] BHQ has been found to be about twice as powerful on an equal weight basis as BHT, and the present results are in general agreement with this observation, 10 mg % appearing to be a suitable concentration in the developing solvent.

BHT absorbs at about 280 mμ and BHQ at about 293 mμ but not significantly at the concentrations required. Nor do they interfere when fatty acid methyl esters prepared from the eluted lipids are subsequently subjected to gas chromatographic analysis. On a polypropyleneglycoladipate column BHT appears as a peak in the C_{12} saturated fatty acid ester region and BHQ as an elevation of the base line in the C_{12}–C_{17} region, but, as normally present at a concentration of only 0.1 % of the esters, no interference is detectable.

WREN AND SZCZEPANOWSKA[7] based their recommendation of the use of BHT on observations that the presence of the antioxidant (a) decreased adsorption losses when a sample of phosphatidylethanolamine which had stood in solution in the laboratory overnight was repeatedly passed through a silica column, and (b) prevented visible tailing of a phosphatidylethanolamine spot re-run after drying for 20 hours in the laboratory on a thin-layer plate. Tests by DR. M. J. FISHWICK in this laboratory, however, show that phospholipids with high peroxide values can still give compact spots with solvents normally used for their separation and that peroxidised phospholipids can still be eluted readily from the plates, as our own results for sample F in Table I also show. It would seem therefore that streaking and low recovery, while undoubtedly capable of detecting advanced autoxidation and polymerization, are not sufficiently sensitive criteria to evaluate the protective action of antioxidants during the important earlier stages of autoxidation.

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