notes on methodology

A lipid-soluble antioxidant from polyallomer centrifuge tubes

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Summary Santowhite, a commercial antioxidant used in the manufacture of polypropylene, contaminates 12-ml polyallomer tubes to the extent of 0.2–0.3 mg/tube. It is distributed through the plastic and appears as a microscopic dust on the tubes' surfaces. In the preparation of polysomes from rat liver by standard methods, approximately 15% of the antioxidant in previously water-washed tubes was removed by suspension of the polysome pellet with stirring in 1–2 ml of Tris buffer, pH 7.8. The polyallomer impurity has been shown to be identical with Santowhite, which is 4,4′-butylidene-bis(6-*tert*-butyl-*m*-cresol), by UV, mass, and NMR spectra. It is not uniformly removed from polyallomer tubes by common detergents but is removed by washing with acetone, to which the plastic is resistant.

Supplementary key words polysomes · Santowhite · lipid analysis

Polyallomer tubes[4] are widely used in the preparation of subcellular organelles by ultracentrifugation. These tubes are composed of a mixture of 95% polypropylene and 5% polyethylene and are translucent, pliable, and resistant to heat (135°C) and a variety of inorganic and organic acids and organic solvents. In the course of studying the lipids that remain attached to ribosomes after standard treatment of rat liver microsomes with 1% deoxycholate and centrifugation through 2.0 M sucrose in polyallomer tubes (1), we noted a novel compound on thin-layer chromatograms of lipid extracted by the method of Folch, Lees, and Sloane Stanley (2) from thick aqueous suspensions of these ribosomes. This compound was identified by mass spectrometry and NMR spectrometry as 4,4′-butylidene-bis(6-*tert*-butyl-*m*-cresol) and was later identified as an antioxidant added as a stabilizer in the manufacture of polypropylene, the plastic that is the main component of polyallomer tubes. This antioxidant is marketed under the trade name Santowhite by the Monsanto Co., St. Louis, Mo.

The purpose of this communication is to warn investigators who might be interested in the study of the lipids of materials centrifuged in polyallomer tubes of the presence of this adherent impurity and to suggest a simple method for its complete removal. Blowing these tubes out with N_2 or washing them with water, 0.5 N KOH, or some common laboratory detergents is not sufficient to remove the antioxidant. Washing twice with acetone, to which polyallomer is resistant, has been found to be the best method of removing the impurity without harming the plastic.

Materials. Santowhite powder was kindly supplied by the Monsanto Co. It was a fine white powder with a mp of 210°C and an empirical formula of $C_{26}H_{38}O_2$. Its UV spectrum was determined in absolute ethanol with a Beckman model DB-GT recording spectrophotometer. The compound has twin maxima at 282 and 286 nm ($E^{1\%}_{1\,cm}$ = 128) with minima at 255 nm ($E^{1\%}_{1\,cm}$ = 20) and 284 nm ($E^{1\%}_{1\,cm}$ = 127). Its mass spectrum showed the molecular ion to be *m/e* 382 with the base peak at 339 (M − 43) representing the loss of the tertiary isopropyl group. The only other major ion was *m/e* 57, signifying cleavage of the isobutyl groups. The 100-MHz NMR spectrum in $CDCl_3$ showed peaks at δ 0.90 (t, 3H, J = 8 Hz), 1.34 (s, 18H), 1.1–2.0 (m, 4H), 2.15 (s, 6H), 4.10 (t, 1H, J = 8 Hz), 4.45 (s, 2H), 6.37 (s, 2H), and 7.07 (s, 2H). The structure of this compound is 4,4′-butylidene-bis(6-*tert*-butyl-*m*-cresol).

The polyallomer ultracentrifuge tubes, ⅝ by 3 inches, were selected randomly from a carton of 50 tubes obtained from Beckman Instruments, Inc. Spectral-analyzed acetone and reagent-grade chloroform were supplied by Fisher Scientific Co. Anhydrous ether was obtained in 1-lb sealed cans from Mallinckrodt Chemical Works (St. Louis, Mo.). Three detergents were used. These were Chemsolv (Mallinckrodt), Joy (Procter and Gamble, Cincinnati, Ohio), and Alconox (Alconox, Inc., New York).

Polysomes were prepared from livers of fasting rats by the method of Wettstein, Staehelin, and Noll (1), as modified by Johnston and Olson (3), using 12-ml polyallomer tubes. The sucrose was extracted with $CHCl_3$. These translucent, straw-colored polysomes were stored at −80°C. They showed maximum UV absorptivity at 259 nm and had a ratio of absorptivity A_{259}/A_{278} of 1.7. On sucrose gradient (15–30%) centrifugation they showed typical polysome profiles and incorporated 1,100 pmoles of [U-^{14}C]leucine into protein per mg of RNA (3).

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[3] Robert E. Olson was a Guggenheim Fellow on leave from St. Louis University in academic 1970–71 during which time part of this work was carried out.

[4] Manufactured by Eastman Chemical Co., Eastman, Tenn., and marketed by Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.

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Procedure. Randomly selected polyallomer tubes were extracted with 10-ml portions of various solvents (water, acetone, 0.5 N KOH) and three commonly used laboratory detergents (Joy, Alconox, and Chemsolv). The solvents were agitated for 1–2 min with a Pasteur pipette. The detergent solutions were prepared from concentrates as follows: Joy, 5% v/v; Chemsolv, 4% v/v; and Alconox, 1% w/v. In the experiments with detergents, the tubes were scrubbed with a nylon-bristle brush for 1–2 min. After this treatment, the tubes were rinsed thoroughly with distilled water and air-dried. 10 ml of acetone was then introduced into the washed tubes and agitated as indicated previously to extract residual Santowhite. The acetone extracts were decanted into a round-bottomed flask and evaporated in vacuo; the solids were dissolved in absolute alcohol, and their UV spectrum was determined. The quantity of Santowhite present was calculated from its $E^{1\%}_{1\,cm}$ = 128 at 286 nm. If the spectrum indicated the presence of any compounds other than Santowhite, the Santowhite was purified by thin-layer chromatography and its estimation by UV absorption was carried out.

Samples of polysomes were thawed at 4°C, suspended in Tris buffer at pH 7.8, and transferred to acid-washed glass tubes for lipid extraction by the method of Folch et al. (2). About 6 mg of total lipid was obtained per 100 mg of ribosomal RNA. The chloroform-soluble lipid from the extraction was chromatographed on 500 mg of silicic acid according to Borgström (4), using 50 ml of chloroform to elute the neutral lipids, 50 ml of acetone to elute any glycolipids, and 50 ml of methanol to elute phospholipids. The neutral lipids were separated by thin-layer chromatography on silica gel G by the method of Mangold and Malins (5), using a solvent system of hexane–diethyl ether–acetic acid 160:40:3. Thin-layer chromatography of polyallomer tube extracts was accomplished by the same method (5).

Results and discussion. Polyallomer tubes (5/8 by 3 inches) with a volume of 12 ml contain 0.2–0.3 mg of acetone-extractable Santowhite. As shown in Table 1, only acetone and the detergent Joy were effective in removing the impurity. Alconox removed only 10% of the Santowhite extractable with acetone, and 0.5 N KOH and Chemsolv removed about 85%. Both Chemsolv and Joy, furthermore, introduced other UV absorbing materials into the tubes. A distilled water rinse, which is used by most molecular biologists in preparing their tubes, with or without a nitrogen gas flush, was ineffective.

TABLE 1. Extraction of Santowhite from polyallomer tubes under various conditions

Treatment	Santowhite Found		Percent Removed
	Before[a]	After	
	mg/tube		
Acetone	0.21	0.01	95
Distilled water[b]	0.22	0.21	9
Chemsolv	0.29	0.04	86
Alconox	0.29	0.26	10
Joy	0.21	0.01	95
KOH (0.5 N)	0.22	0.04	82

[a] The control values were obtained by acetone extraction.
[b] The prior use of N_2 to flush out any particulate matter did not affect the result of water extraction.

When polysomes were prepared in polyallomer tubes and the pellet was suspended in 1–2 ml of Tris buffer at pH 7.8 by agitating with a bone spatula, about 15% of the Santowhite of the tube (0.03–0.04 mg) was taken up. When this aqueous suspension of polysomes was subsequently extracted with chloroform–methanol 2:1 and the neutral lipids were chromatographed on silica gel G, spots appeared corresponding to cholesteryl ester, triglycerides, Santowhite, and free fatty acids. The R_F of Santowhite in hexane–diethyl ether–acetic acid 160:40:3 was 0.22. It generally has been assumed that the deoxycholate used to destroy the membrane of the rough reticulum eliminates all lipids from the pelleted polysomes, particularly when they are spun through a layer of chloroform-extracted 2.0 M sucrose. It is obvious from this work, however, that 8% of the original lipid of the membrane remains attached to ribosomes and possibly promotes the solubility of Santowhite in the ribosomal slurry.

There is no doubt about the identity of the polyallomer tube impurity and Santowhite. The R_F of the impurity in the solvent system used and its UV, mass, and NMR spectra were identical with those of authentic Santowhite.

Finding the polyallomer antioxidant Santowhite in our lipid extracts of ribosomes only illustrates the importance of constant surveillance of vessels involved in the preparation of biological materials destined for lipid analysis, particularly plastics. The fact that the vulnerable step in our procedure involved an *aqueous* suspension of largely defatted materials in polyallomer tubes only serves to emphasize this important precaution.

This research was supported in part by grant AM 09992 from the National Institutes of Health, USPHS, and by a grant from the Hoffmann-La Roche Co., Basel and Nutley. Dr. Robert E. Olson thanks the Guggenheim Foundation, New York, for fellowship support during a period of academic leave from St. Louis University (1970–71).

The authors express their appreciation to Dr. W. Vetter and Dr. G. Englert of the Hoffmann-La Roche Company, Basel, for the mass and NMR spectra reported herein. The expert technical assistance of M. Kobel, U. Timm, and R. Houser is also gratefully acknowledged.

Manuscript received 22 August 1973; accepted 10 January 1974.

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