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Chromatographic separation of allylic alcohols on silicic acid columns: Analysis of the nonsaponifiable lipids of an ascites tumor derived from a benzpyrene-induced sarcoma*

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SUMMARY

Chromatographic separations of dimethyl allyl alcohol, geraniol, nerol, nerolidol, and *trans-trans*-farnesol were achieved on silicic acid–Super Cel columns. Application of this chromatographic system to the radioactive nonsaponifiable lipids formed from mevalonic acid-C^{14} by an ascites tumor is described.

Silicic acid column chromatography has proved very useful in the separation and isolation of many of the sterols found in the nonsaponifiable fraction of lipid mixtures (1–8). In the course of an investigation of the nonsaponifiable lipids formed from mevalonic acid-C^{14} by a rat ascites tumor, a highly radioactive fraction was isolated by chromatography on silicic acid. This fraction was shown to contain a large amount of radioactive *trans-trans*-farnesol. This finding stimulated an investigation of the chromatographic behavior of some other allylic alcohols on silicic acid columns.

MATERIALS

DL-Mevalonic acid-2-C^{14} (0.2 μc/μmole) was prepared by alkaline hydrolysis of the lactone (purchased from the Radiochemical Centre, Amersham, Bucks, England) after dilution with purified, unlabeled lactone. *trans-trans*-Farnesol-2-C^{14} was a sample previously prepared by chemical synthesis (9). Five minor contaminants were detected upon chromatography on silicic acid columns, which were prepared as described below. Dimethyl allyl alcohol-1-H^3 was prepared by reduction of 3-methyl crotonic acid with tritium-labeled lithium aluminum hydride (10). A minor contaminant was demonstrated by both gas-liquid chromatography and silicic acid column chromatography. Nerolidol-2-C^{14} was isolated by column chromatography on silicic acid after treatment of farnesyl pyrophosphate-2-C^{14} (9) with dilute acid. Geraniol-1-H^3 and its *cis*-isomer, nerol-1-H^3, were prepared by reducing citral with tritium-labeled lithium aluminum hydride. The citral was prepared by oxidation of geraniol with manganese dioxide (11). Upon gas-liquid chromatography, two peaks were noted, corresponding in their retention times to *trans*-citral (91%) and *cis*-citral (9%).

EXPERIMENTAL METHODS

The preparation of the silicic acid columns used in this study was carried out as described by Frantz et al. (1). Silicic acid (Malinckrodt Chemical Works, 100 mesh, analytical reagent, "suitable for chromatographic analysis by the method of Ramsey and Patterson") and Hyflo Super Cel (Johns Manville Corporation) were thoroughly mixed in a beaker in a 2 : 1

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ratio (by weight), respectively. A slurry in benzene (reagent grade, thiophene-free) was poured into glass columns, and the columns were packed under a nitrogen pressure of 5–10 lb/in^2. The samples were applied to the column in a small volume of benzene (1–2 ml), and they were eluted with benzene. Unless otherwise stated, fractions 5 ml in volume were collected, using an automatic fraction collector. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Recovery of cholesterol from the counting fluid as the digitonide, recovery of cholesterol from the digitonide, purification of cholesterol by way of the dibromide, and colorimetric measurement of cholesterol were carried out by methods previously described (3). Gas-liquid radiochromatography of the labeled allylic alcohols was performed as described previously by Popják et al. (12–14). Purification of squalene by way of the thiourea adduct was carried out as described by Goodman and Popják (15).

A rat ascites tumor (20 g, fresh weight) derived from a benzpyrene-induced sarcoma was, after preliminary washings to remove red blood cells, incubated for 2 hr with DL-mevalonic acid-2-C^{14} (200 μmoles) in 0.05 M potassium phosphate buffer, pH 7.4, containing 75 mM KCl and 1.5 mM $MgSO_4$ as described previously (16). The nonsaponifiable fraction contained 9.56 $\times$ 10^6 cpm (corresponding to an incorporation of 28.3 μmoles of mevalonic acid into the nonsaponifiable material). A portion of this material was applied to a silicic acid column, 1 cm in diameter and 100 cm in length.

RESULTS

Figures 1 and 2 illustrate separations of known radioactive allylic alcohols on silicic acid columns. The radioactivity in the dimethyl allyl alcohol, nerol, and geraniol areas was determined by analyzing aliquots of the fractions eluted from the column. The identity of each of the chemically synthesized radioactive allylic alcohols was confirmed by gas-liquid radiochromatographic analysis after elution from the silicic acid columns.

After several incubations of mevalonic acid-C^{14} with the ascites tumor, the distribution of radioactivity in the nonsaponifiable material prompted us to carry out a large-scale incubation in order to obtain sufficient radioactive material for further analytical studies. Figures 3 and 4 show the chromatogram of the material obtained from this incubation. The largest peak (center at fraction 166) contained approximately 50% of the radioactivity eluted from the column. Preliminary degradative studies of this material indicated the probable presence of a C^{14}-labeled isopropylidene group. Further studies indicated that less than 1% of the radioactivity in this material was precipitable with digitonin (in the presence of added carrier cholesterol) under the conditions described by Sperry and Webb (17). Upon gas-liquid radiochromatographic analysis (with unlabeled dimethyl allyl alcohol, geraniol, nerolidol, *cis-trans*-farnesol, and *trans-trans*-farnesol), the radioactivity emerged from the column with the *trans-trans*-farnesol.

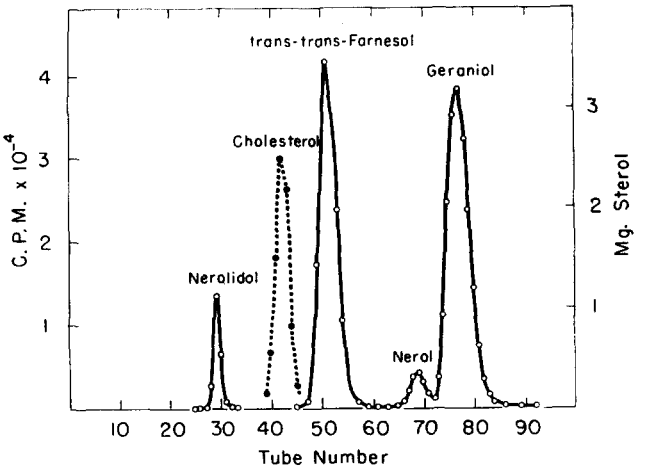

FIG. 1. Chromatographic separation of radioactive prenols and unlabeled cholesterol. O——O, radioactivity; ●- - -●, cholesterol measured colorimetrically; column dimensions: 1 $\times$ 50 cm.

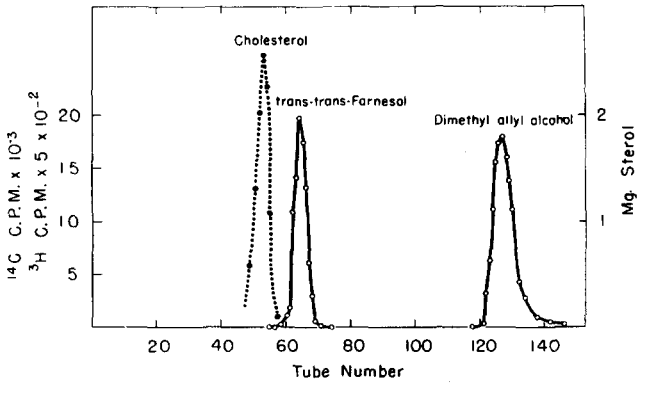

FIG. 2. Chromatographic separation of cholesterol, farnesol-C^{14}, and dimethyl allyl alcohol-H^3. Symbols are the same as in Fig. 1. Column dimensions: 1 $\times$ 52 cm; size of fractions: 4.5 ml.

Cholesterol (peak at fraction 122 as judged by colorimetric assay) was eluted shortly after an unidentified, highly radioactive substance (peak at fraction 116). That radioactive cholesterol was formed by the tumor is indicated by the following experiment. The material in fractions 119 through 127 was treated with digitonin under the conditions described by Sperry and Webb (17). After recovery of the sterol from the digitonide and subsequent dilution with purified, unlabeled

cholesterol, the cholesterol was further purified by way of the dibromide. After purification of the cholesterol by way of the digitonide, no change in the specific activity of the cholesterol was observed upon further purification by way of the dibromide. The specific activity of the cholesterol before and after the dibromide purification was 1,130 and 1,140 cpm/mg, respectively.

Radioactive material corresponding to the first peak shown in Fig. 3 (center at fraction 18) was diluted with purified, unlabeled squalene and purified twice by way of the thiourea adduct. No significant change in the specific activity of the squalene was observed (Table 1). However, as it is possible that other hydrocarbons might show similar properties, definite assignment of the structure of squalene to this radioactive component cannot be made on the basis of this evidence alone.

TABLE 1. PURIFICATION OF SQUALENE BY WAY OF THE THIOUREA ADDUCT

	Specific Activity
	cpm/mg × 10^{-2}
Initial	1.9
After first purification	1.8
After second purification	1.8

DISCUSSION

Effective separations of the allylic alcohols used in this study can be most quickly achieved by the use of gas-liquid chromatography (12). A significant advantage of the silicic acid method is the greater capacity of these columns. While a detailed study of the capacity of silicic acid columns with respect to these prenols was not made, no overloading of the column was observed with approximately 5 mg of farnesol on a column with a diameter of 1 cm. Possible uses of this column include (1) purification and isolation of relatively large quantities of these prenols from natural sources, (2) purification of a given allylic alcohol from by-products arising during its chemical synthesis, and (3) application to studies of the chemical nature of the products arising from the treatment of these isoprenoid alcohols with Lewis acids.[1]

With regard to the use of this type of column for the purification and isolation of these prenols from animal tissues, several additional comments must be made. Farnesol and geraniol have elution volumes greater than

[1] Exposure of pure *trans-trans*-farnesol to 5 N HCl (2 ml) in ethanol (4 ml) for 90 min at 70° resulted in the recovery of at least seven components when the products were subjected to chromatography on silicic acid. These components were not formed from farnesol after treatment with strong alkali under similar conditions.

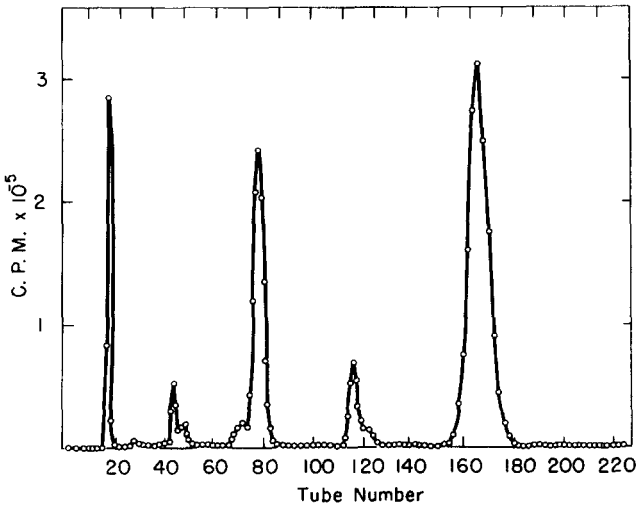

FIG. 3. Chromatogram of C^{14}-labeled nonsaponifiable material from ascites tumor. Symbols are the same as Fig. 1.

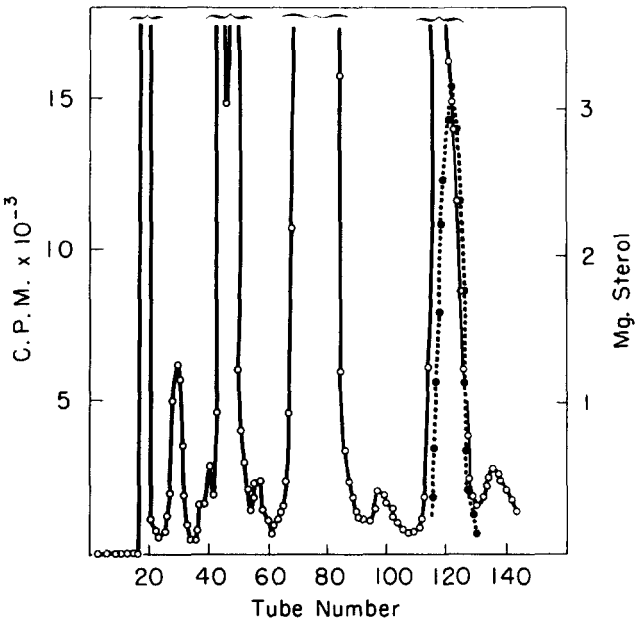

FIG. 4. Chromatogram of C^{14}-labeled nonsaponifiable material from ascites tumor. (Same as in Fig. 3 but with a 100-fold change in the scale of the ordinate.) Symbols are the same as in Fig. 1.

that of cholesterol. Among the sterols that also show greater elution volumes are Δ^7-cholestenol (4, 7), Δ^8-cholestenol (3), $\Delta^{5,7}$-cholestadienol (4, 7), $\Delta^{5,24}$-cholestadienol (2, 8), and $\Delta^{8,24}$-cholestadienol (3). Separation of these sterols from farnesol and geraniol can be effected by precipitation of the sterols with digitonin. Nerolidol shows an elution volume smaller than that of cholesterol. Lanosterol (4, 6), 24,25-dihydrolanosterol (4, 6), 4α-methyl-Δ^8-cholestenol (4, 6), and 4α-methyl-Δ^7-cholestenol (5) are among the sterols that

also show elution volumes smaller than that of cholesterol. Since the introduction of a methyl group at position 4 reduces the ease of precipitation of these sterols with digitonin, complete separation of these sterols from nerolidol would not readily be accomplished through the use of digitonin precipitation.

The magnitude of the formation of radioactive farnesol from mevalonic acid by the ascites tumor cells is quite remarkable. Factors leading to its accumulation by the tumor, under the incubation conditions employed, have been discussed previously (16).

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