Evidence for the existence of cholesteryl alk-1-enyl ethers in bovine and porcine cardiac muscle

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ABSTRACT Evidence is presented for the existence of cholesteryl alk-1-enyl ethers in bovine and porcine cardiac muscle. Several different fatty chains are present in the cholesteryl ethers, the major species having 16 and 18 carbon atoms. The cholesteryl alk-1-enyl ether concentration was found to be 0.08 and 0.01 μmoles/100 mg of neutral lipid in bovine and porcine cardiac muscle, respectively.

SUPPLEMENTARY KEY WORDS neutral lipid · thin-layer chromatography · gas–liquid chromatography · silicic acid column chromatography · infrared

ALDEHYDOGENIC LIPIDS were first observed in mammalian tissues in 1929, and they were termed plasmalogens (1). Later the aldehydogenic nature of this type of lipid was shown to be due to the presence of an alkali-stable, labile-acid alk-1-enyl ether of glycerol. These glyceryl ethers occur primarily as acylated neutral lipids or phospholipids (2, 3).

More recently the aldehydogenic lipids in mammalian tissues have been shown to include free fatty aldehydes (4). The purpose of this paper is to extend the study of the aldehydogenic lipids and present data which establish the existence of cholesteryl alk-1-enyl ethers as naturally occurring lipids.

EXPERIMENTAL PROCEDURES

Extraction of Lipids

Bovine and porcine hearts were obtained from a slaughterhouse and were stored in ice during transit. Pericardial fat was removed in the cold, and the chambers of the heart were opened and rinsed with 0.9% NaCl. Lipids were extracted by homogenizing the wet tissue in a Waring Blendor with chloroform–methanol 2:1 (5). The extract was filtered through Whatman filter paper No. 1, and the filtrate was shaken with a volume of 1% NaCl equal to one quarter of the filtrate volume. The phases were allowed to separate, and the lower phase was dried over anhydrous Na_2SO_4. The lipid extract was then concentrated under vacuum on a rotary evaporator at 33°C, blanketed with nitrogen, and diluted to a final volume. Duplicate aliquots of the lipid extracts were dried under nitrogen and weighed to determine the total lipid. A total of 145.60 g of lipid was extracted from 2.748 kg of bovine cardiac muscle, and 4.55 g of lipid was extracted from 0.120 kg of porcine cardiac muscle.

Isolation of Cholesteryl Alk-1-enyl Ethers

Since the amount of lipid isolated from bovine cardiac muscle was appreciably greater than that prepared from porcine heart, different procedures were used to isolate the cholesteryl alk-1-enyl ethers.

Bovine Cardiac Muscle

Preparation of Unsaponifiable Lipids. Phospholipids were removed from the total lipid extract by flocculation with acetone and $MgCl_2$ (6). This flocculation procedure was repeated twice more, and the solvents were removed from the lipids as before. A total of 23.50 g of neutral lipid and 121.31 g of phospholipid was obtained.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

An aliquot containing 22.00 g of neutral lipid was saponified by refluxing for 2 hr with an excess of 0.5 N ethanolic sodium hydroxide. The saponification mixture was diluted with water, cooled to room temperature, and the unsaponifiable lipids were extracted with diethyl ether. The ether solution was dried over anhydrous Na_2SO_4, concentrated under reduced pressure, and the unsaponifiable lipids were dissolved in *n*-heptane. Duplicate aliquots were weighed; 2.94 g of unsaponifiable material was obtained.

Column Chromatography. The unsaponifiable lipid, 2.72 g, was applied in equal amounts to 10 chromatographic columns, 2 cm I.D., each containing 18 g of silicic acid in *n*-heptane (7). The first two fractions, I (1–50 ml) and II (50–180 ml), were eluted with 1% ethyl ether in *n*-heptane. Previously it had been demonstrated that cholesteryl alkyl ethers could be expected to be found in fraction II (8). The third fraction (III) was eluted with 225 ml of 1% ethyl ether in *n*-heptane and then with 60 ml of 4% ethyl ether in *n*-heptane. The remaining lipid was eluted with 500 ml ethyl ether. Each fraction was concentrated to dryness on a rotary evaporator, blanketed with nitrogen, and dissolved in a known volume of chloroform. Aliquots of each fraction were weighed; 2.64 g of unsaponifiable lipid was recovered from the silicic acid columns.

TLC. The method of preparing thin layers of Silica Gel G and aluminum oxide and the solvent systems used to fractionate neutral lipid mixtures into individual lipid types on these plates have been described before (4, 8–10).

Following separation of the unsaponifiable lipids by column chromatography, the lipid present in each fraction was analyzed by TLC. By this procedure each column fraction was monitored for compounds migrating the same distance as the cholesteryl alkyl ether standard, and the cholesteryl ethers were separated from other lipids. Aliquots from each column fraction were applied to neutral Silica Gel G plates, and the chromatogram was developed with hexane–chloroform–methanol 73.5:25:1.5. Mixed standards were applied to a separate region of the same plate. Once the development was complete the chromatograms were air-dried, sprayed with rhodamine 6G in 80% methanol, and viewed under ultraviolet (UV) light. The area of the plate containing lipid migrating as the cholesteryl alkyl ether standard was scraped off, and the lipid was eluted with chloroform. When further chemical analyses were not required, the lipids were visualized with iodine vapor.

Porcine Cardiac Muscle

Column Chromatography. An aliquot containing 3.28 g of total lipid from porcine cardiac muscle was applied to silicic acid columns previously equilibrated with *n*-heptane (7). The columns were developed with 250 ml of 1% ethyl ether in *n*-heptane, and a lipid mixture containing cholesteryl ethers was eluted. The remaining neutral lipids were eluted with chloroform. Each column fraction was concentrated under vacuum, and the lipid was weighed. A total of 1.6 g of neutral lipid was obtained.

TLC. The lipid mixture eluted from the silicic acid columns with 1% diethyl ether in *n*-heptane was further separated on neutral Silica Gel G plates developed with hexane–chloroform–methanol 73.5:25:1.5. The lipid migrating the same distance as the cholesteryl alkyl ether standard was isolated and was separated a second time on thin layers of neutral Silica Gel G using hexane–chloroform 75:25 as the developing solvent. Chromatography using these two solvent systems separates the cholesteryl ethers from a number of contaminants, but it does not separate them from the cholesteryl esters. Final resolution of the cholesteryl ethers from the esters was achieved on a TLC plate of aluminum oxide H developed with hexane–benzene 90:10.

GLC and Mass Spectrometry

The fatty aldehydes released by acid hydrolysis of the cholesteryl alk-1-enyl ethers were converted to dimethyl acetals (11). The pure dimethyl acetals were obtained by TLC on neutral Silica Gel G plates using hexane–chloroform 75:25 as the developing solvent (4).

The instrument employed here and the conditions used to identify the dimethyl acetals have been described before (4).

The same instrument was used to identify cholesterol, and columns containing 2.3% SE 30 on acid-washed, silanized 100–120 mesh Gas-Chrom S were used. The column operating temperature was 230°C with a gas flow rate of 112 ml/min and an inlet pressure of 40 psi.

The retention times of standard and sample dimethyl acetals were calculated relative to that of the dimethyl acetal of octadecanal. Identification was based on a comparison of the observed relative retention times to those obtained with standards chromatographed under identical conditions.

Cholesterol was identified by comparing its retention time to that of an authentic sample and by cochromatography of the unknown lipid with cholesterol.

Combined GLC–mass spectrometry using an F & M model 400 gas chromatograph, equipped with a 2.3% SE 30 column, and an LKB 9000 single focusing spectrometer was also employed to further establish the identification of the major dimethyl acetals and cholesterol.

Other Analytical Procedures

Acid hydrolysis of the cholesteryl alk-1-enyl ethers isolated from bovine cardiac muscle was carried out by heating 5 mg of lipid with 5 ml of 90% acetic acid at

37°C for 17 hr (12). The lipid was extracted with chloroform, washed with 2 M KCl and water, and then dried over anhydrous Na_2SO_4. The solvent was removed under nitrogen and the residue was dissolved in chloroform prior to TLC. Cholesteryl alk-1-enyl ethers obtained from porcine cardiac muscle were hydrolyzed under the conditions used in the hydrazone analysis (13) reduced in scale by a factor of two. In this hydrolysis 95% ethanol was substituted for the ethanolic hydrazine solution. The lipid extract was concentrated under nitrogen, and the residue was dissolved in chloroform prior to TLC.

Fatty aldehydes were quantitated as *p*-nitrophenylhydrazones (13), and cholesterol was determined with the Liebermann-Burchard reagent (14).

Infrared spectra of the isolated ether fraction before and after acid hydrolysis were obtained on a Beckman IR8 instrument (Beckman Instruments, Inc., Fullerton, Calif.) and compared to the spectrum of the cholesterol alkyl ether standard.

In an attempt to exclude extraneous compounds, a blank representing the solvents used for lipid extraction, flocculation, saponification, and column chromatography was prepared in each instance, concentrated, and adjusted to a final volume. When this concentrate was analyzed by TLC, no lipid residue below the solvent front was detected upon staining with iodine vapor. GLC of the concentrate under the conditions used to identify dimethyl acetals indicated the presence of several components which appeared on the descending side of the solvent peak. Any components resembling these in the natural mixtures were not considered. Analysis of aliquots of this concentrate for cholesterol and fatty aldehydes gave negative results.

Materials

All solvents were reagent grade and, with the exception of methanol, were redistilled before use. The solid support and liquid phases for GLC were obtained from the Applied Science Laboratories Inc. (State College, Pa.). Fatty aldehyde standards were purchased from the Aldrich Chemical Co. Inc. (Milwaukee, Wis.). Mallinckrodt silicic acid, 100 mesh, was used in the column chromatographic procedures.

Synthetic cholesteryl alkyl ethers were prepared as described before (8).

RESULTS

TLC

Bovine Cardiac Muscle. As indicated in the text, 2.64 g of unsaponifiable neutral lipid was recovered from an initial 2.72 g applied to the silicic acid columns, indicating that the recovery of lipid was essentially quantitative.

The lipids in the first three column fractions were concentrated and further fractionated by TLC using hexane–chloroform–methanol 73.5:25:1.5 as the developing solvent (4). Of the three fractions, only fraction II contained a compound which migrated the same as the standard cholesteryl hexadecyl ether. This component was isolated, diluted to a known volume, and an aliquot was subjected to mild acid hydrolysis (12). Fig. 1

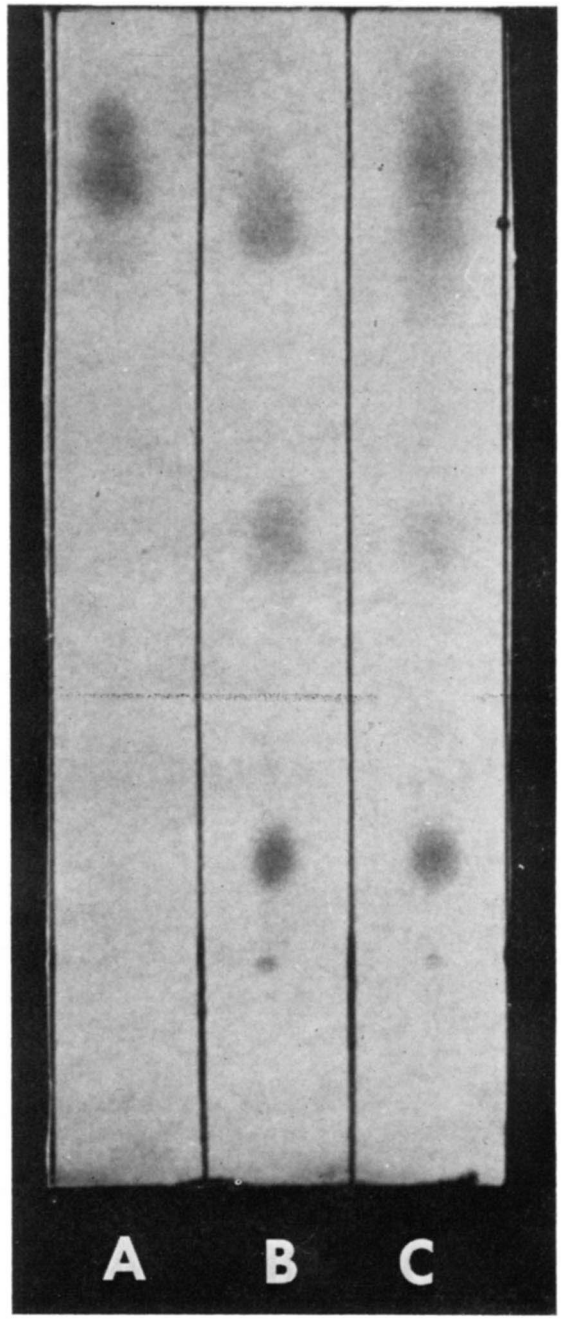

FIG. 1. Thin-layer chromatogram on neutral Silica Gel G of the nonpolar unsaponifiable neutral lipids from bovine cardiac muscle eluted from a silicic acid column by 1% diethyl ether in *n*-heptane. Solvent, hexane–chloroform–methanol 73.5:25:1.5; indicator, iodine vapor. *A*, unsaponifiable lipid prior to acid hydrolysis; *B*, mixed standards from origin to front-cholesterol, octadecanal and cholesteryl hexadecyl ether; *C*, unsaponifiable lipid after mild acid hydrolysis.

is a thin-layer chromatogram of this component before and after acid hydrolysis. Prior to acid hydrolysis, two incompletely separated components were observed, both migrating with a mobility similar to the cholesteryl ether standard. No components with a chromatographic mobility less than the synthetic cholesteryl ether standard were apparent; thus the unhydrolyzed lipid does not contain free fatty aldehydes or cholesterol. TLC of the lipid before and after acid hydrolysis in a second solvent system, isobutanol–hexane–methanol 3:100:3, gave results similar to those in Fig. 1.

Following acid hydrolysis two new components appeared, one with the chromatographic mobility of cholesterol, and the other moving as the octadecanal standard. An acid-stable material which moved as the cholesteryl alkyl ether standard was still present.

The components present after acid hydrolysis were isolated by TLC using hexane–chloroform–methanol 73.5:25:1.5 as the developing solvent. Analysis of the material with a chromatographic mobility similar to the free fatty aldehydes as *p*-nitrophenylhydrazones indicated 18.2 μmoles of aldehyde; analysis of the component migrating as cholesterol indicated 17.8 μmoles of this sterol. This gives a molar ratio of fatty aldehyde to cholesterol of 1.03. For a cholesteryl alk-1-enyl ether a molar ratio of 1.00 would be expected.

This lipid was isolated from 23.5 g of neutral lipid; thus, based on the hydrazone analysis, the concentration of cholesteryl alk-1-enyl ethers in bovine cardiac muscle is 0.08 μmoles/100 mg of neutral lipid.

Porcine Cardiac Muscle. The lipid mixture eluted from the silicic acid columns with 1% ethyl ether in *n*-heptane was initially chromatographed on neutral Silica Gel G plates with hexane–chloroform–methanol 73.5:25:1.5 as the developing solvent to separate the cholesteryl ethers from the free fatty aldehydes which are present in this column fraction. Further separation of the cholesteryl ethers from hydrocarbons such as squalene and other nonpolar lipids was achieved by TLC on neutral Silica Gel G plates developed with hexane–chloroform 75:25. Final resolution of the cholesteryl ethers from the cholesteryl esters was achieved by chromatography on thin layers of aluminum oxide with hexane–benzene 90:10 as the developing solvent. Fig. 2 is a typical chromatogram showing the resolution of the cholesteryl ethers from the cholesteryl esters in this latter solvent system.

Fig. 3 is a thin-layer chromatogram of the purified cholesteryl ether fraction before and after acid hydrolysis. Prior to acid hydrolysis only one component which migrated as the cholesteryl ether standard was present. After acid hydrolysis two new components were present, one migrating as cholesterol and the other as octadecanal. An acid-stable component was also present, and it migrated as the cholesteryl alkyl ether standard. Analysis of an aliquot of the purified cholesteryl ether fraction for aldehydogenic lipids (13) indicated a total of 0.156 μmoles. Since this lipid was isolated from 1.6 g of neutral lipid a cholesteryl alk-1-enyl ether concentration of 0.01 μmoles/100 mg of natural lipid is indicated.

Gas–Liquid Chromatography

The lipid components which after acid hydrolysis had the same chromatographic mobility as the free fatty aldehyde and cholesterol standards were analyzed by GLC.

The gas chromatographic tracing of the dimethyl acetals from bovine cardiac muscle is presented in Fig. 4. The dimethyl acetals were identified by comparing their retention times relative to that of the dimethyl acetal of octadecanal with standard values observed on the same column and with values in the literature (15). The relative retention times for standard dimethyl acetals on an Apiezon M column are presented in Table 1. Peaks numbered 8, 11, 13, and 14 in Fig. 4 correspond to the 15:0, 16:0, 17:0 and 18:0 dimethyl acetals and account for 80% of the total components present. The dimethyl acetals of hexadecanal and octadecanal represent 43% and 28%, respectively, of the total.

The dimethyl acetals from porcine cardiac muscle were also analyzed by GLC and were identified in a similar manner. Compounds ranging in chain length from 12 to 18 carbon atoms were present; the 15:0 and 16:0 dimethyl acetals were the major components.

TABLE 1 RELATIVE RETENTION TIMES ON APIEZON M OF DIMETHYL ACETALS FROM CHOLESTEROL ALK-1-ENYL ETHERS FROM BOVINE CARDIAC MUSCLE

Peak Number	Short Hand Designation*	Apiezon M		
		Natural Mixture	Standards	Literature (15)
1	10:0	0.046	0.045	
2	12:0	0.092	0.090	
3	U†	0.116		
4	13:0	0.130		
5	U	0.144		
6	14:0	0.176	0.179	0.180
7	U	0.228		0.224
8	15:0	0.268		0.272
9	U	0.316		
10	16:1	0.356		0.361
11	16:0	0.420	0.420	0.424
12	17:0 br	0.565		0.567
13	17:0	0.648		0.646
14	18:0	1.000	1.000	1.000

All retention times are relative to the dimethyl acetal of octadecanal. Identification of the dimethyl acetal designated 13:0 was obtained from a plot of the log of the relative retention time of standard dimethyl acetals versus their molecular weight.

* For the corresponding dimethyl acetal; no. of carbon atoms: no. of double bonds, br, branched.

† U denotes an unidentified compound.

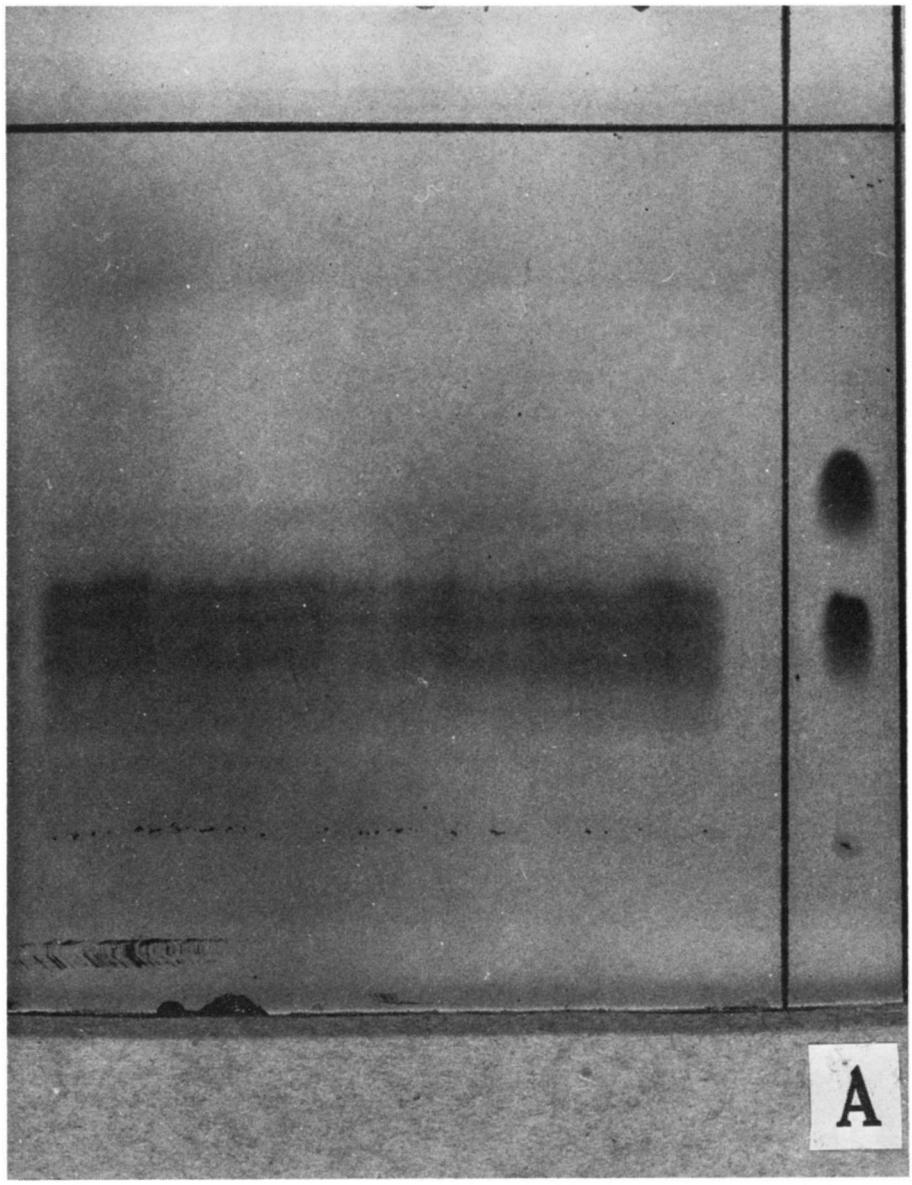

FIG. 2. Thin-layer chromatogram on Aluminum Oxide H of the mixed cholesteryl ether-cholesteryl ester fraction from porcine cardiac muscle isolated from Silica Gel G layers. Solvent hexane–benzene 9:1; indicator, iodine vapor. Standards in column *A* from origin to front-cholesteryl oleate and cholesteryl hexadecyl ether.

The lipid component which after acid hydrolysis had the same mobility as cholesterol during TLC in each instance was isolated and analyzed on a 2.3% SE 30 column at 230°C. The retention time for standard cholesterol and the lipid in question were identical, and cochromatography gave one symmetrical peak.

Infrared and Mass Spectra

In Fig. 5 spectrum *A* is that of the lipid component present in silicic acid column fraction II from bovine cardiac muscle which migrated during TLC as did the synthetic cholesteryl ether. This spectrum was taken prior to mild acid hydrolysis. Spectrum *B* is that of the lipid component present in fraction II which after acid hydrolysis again migrated as did the synthetic cholesteryl alkyl ether. Spectrum *C* is that of a synthetic cholesteryl alkyl ether.

All three spectra exhibit absorption bands characteristic of the hydrocarbon structure of the molecule at 2930 and 2855 cm^{-1}, 1469–1466 cm^{-1}, 1445–1442 cm^{-1}, and near 1378–1367 cm^{-1}. The carbonyl stretching absorption in the range 1747–1744 cm^{-1} is present in spectra *A* and *B*. This is a strong absorption and indicates that the natural lipid is not comprised exclusively of cholesteryl ethers. The absorption at 1116 cm^{-1} found in all three spectra is the C–O frequency characteristic of the ether group.

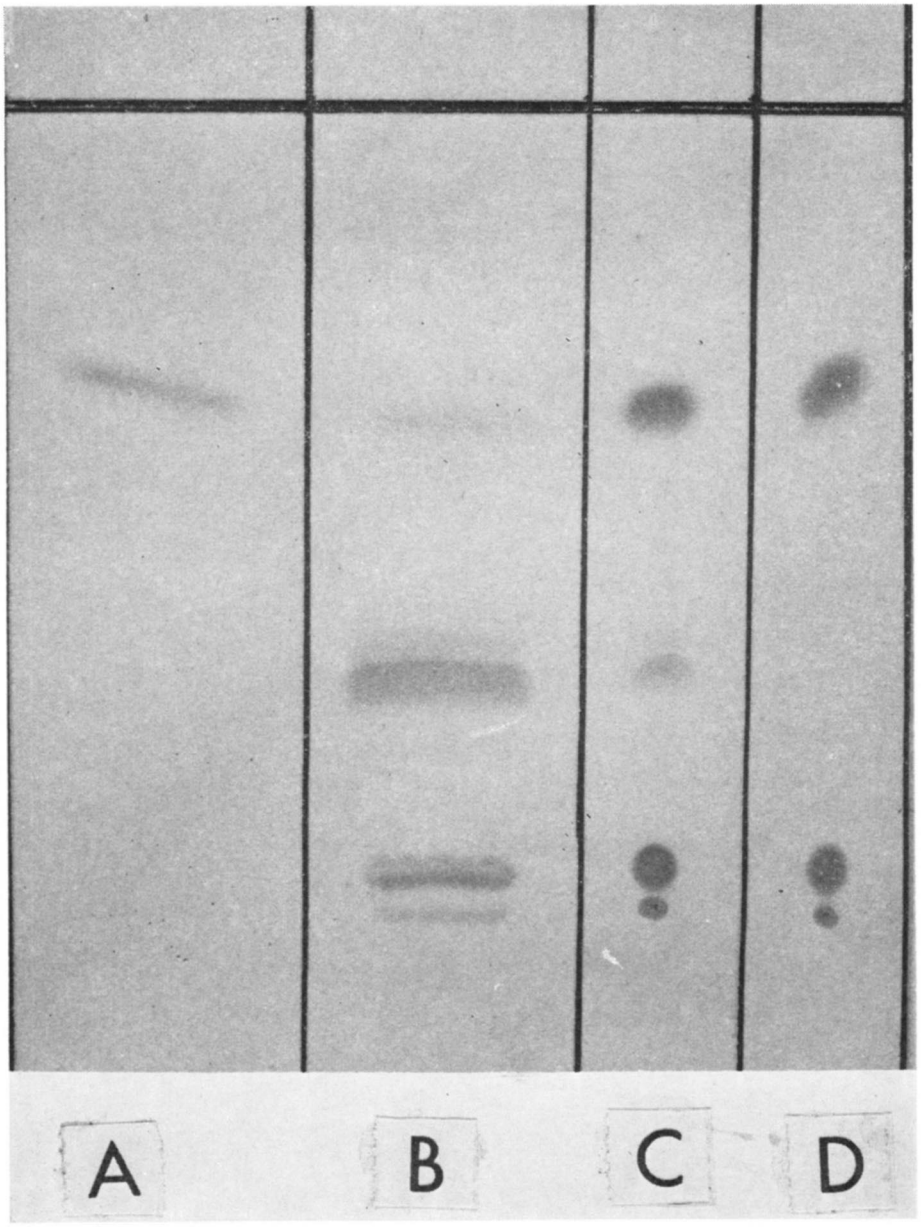

FIG. 3. Thin-layer chromatogram on neutral Silica Gel G of the purified cholesteryl ether fraction from porcine cardiac muscle isolated from Aluminum Oxide H layers. Solvent hexane–chloroform–methanol 73.5:25:1.5; indicator, iodine vapor. *A*, cholesteryl ether fraction before acid hydrolysis; *B*, cholesteryl ether fraction after acid hydrolysis; *C*, mixed standards, from origin to front-cholesterol, octadecanal, and cholesteryl hexadecyl ether; *D*, cholesterol and cholesteryl hexadecyl ether.

The band most characteristic of the alk-1-enyl ether grouping is the absorption near 1668–1666 cm^{-1} in spectrum *A* indicating the presence of a —CH=CH—O— grouping (16, 17). This absorption is absent from spectrum *B* since the vinyl ether linkage is destroyed by mild acid hydrolysis and would not be present in spectrum *C* obtained from the cholesteryl alkyl ether.

The lipid component present after acid hydrolysis of the bovine cardiac lipid which chromatographed by TLC and GLC as cholesterol was found to have an infrared spectrum identical to cholesterol.

The mass spectra of the principal dimethyl acetal peaks, numbers 11 and 14, Fig. 4, were superimposable on the spectra of the dimethyl acetals of hexadecanal and octadecanal. The mass spectrum of the compound identified as cholesterol was also found to be superimposable on that of an authentic standard.

DISCUSSION

Previously data were presented to establish the existence of cholesteryl alkyl ethers as naturally occurring neutral

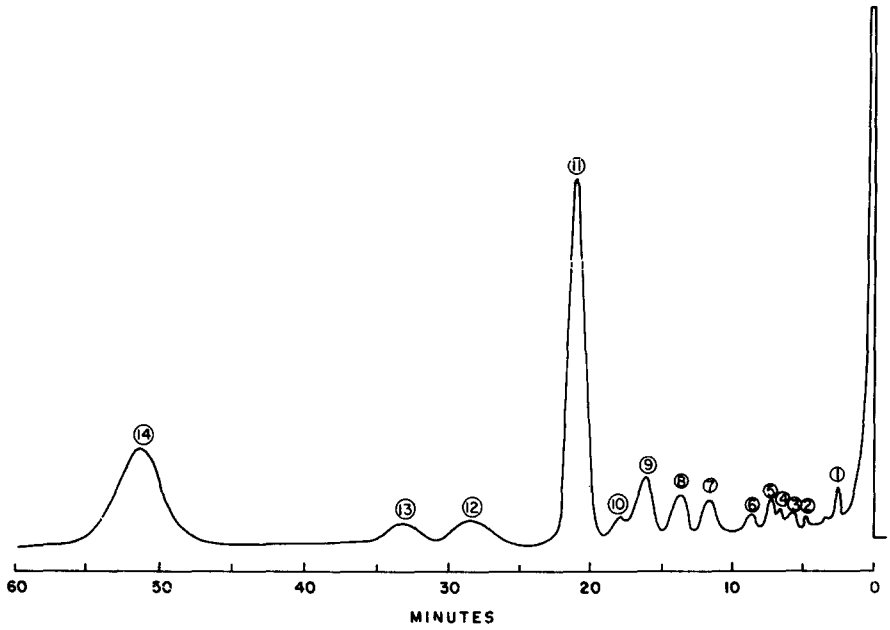

FIG. 4. Gas–liquid chromatogram. Aldehyde dimethyl acetals isolated following mild acid hydrolysis of the nonpolar unsaponifiable neutral lipids from bovine cardiac muscle eluted from a silicic acid column by 1% ethyl ether in *n*-heptane. Liquid phase, 15% Apiezon M on alkali washed, silanized 80–100 mesh Gas-Chrom S. Chain lengths and double bonds: 1, 10:0; 2, 12:0, 4, 13:0; 6, 14:0; 8, 15:0; 10, 16:1; 11, 16:0; 12, 17:0 (iso or anteiso); 13, 17:0; 14, 18:0. Peaks numbers 3, 5, 7, 9 remain unidentified. (2.3% SE 30 can also be used as the liquid phase.)

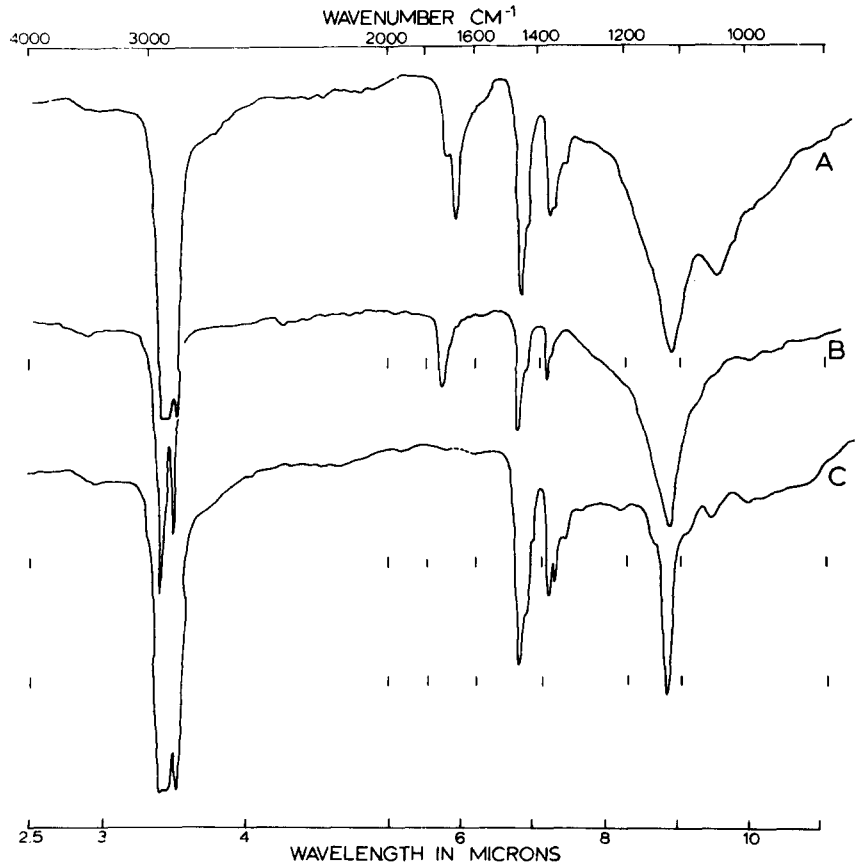

Fig. 5. IR spectra; *A*, unsaponifiable neutral lipid from bovine cardiac muscle isolated by column chromatography and TLC; *B*, unsaponifiable lipid after mild acid hydrolysis; *C*, synthetic cholesteryl hexadecyl ether. Solvent CCl_4 in an NaCl cell.

lipids in bovine cardiac muscle (8). The data reported here establishes that cholesteryl alk-1-enyl ether is a natural lipid component of both bovine and porcine cardiac muscle.

Two different procedures were employed to isolate the cholesteryl alk-1-enyl ethers. The initial method designed to isolate large amounts of cholesteryl alk-1-enyl ethers from bovine cardiac muscle required considerable volumes of solvent and treatment of the neutral lipid with alkali. Since in this procedure the possibility existed that the TLC results obtained might reflect in part the method of isolation which was employed, a different procedure was utilized with the lipid from porcine cardiac muscle. In this instance the cholesteryl alk-1-enyl ethers were isolated by methods that did not require large volumes of solvent or treatment with alkali. Because reagent blanks prepared from the same solvents did not indicate the existence of artifacts on TLC, and since the chromatographic results were essentially the same in both preparations, it is felt that the TLC data obtained reflect the existence of cholesteryl alk-1-enyl ethers in lipid extracts of both bovine and porcine cardiac muscle. Further support for this conclusion is that the lipids which arise after acid hydrolysis of the purified cholesteryl ether were identified by independent techniques as cholesterol and fatty aldehydes. In addition, the absorption band characteristic of an alk-1-enyl ether is present in the infrared spectrum of the purified lipid from bovine heart before but not after mild acid hydrolysis, and essentially equivalent molar amounts of cholesterol and fatty aldehydes were found upon analysis of the purified lipid.

It is interesting to note that the fatty chains of the cholesteryl alk-1-enyl ethers isolated from bovine cardiac muscle are primarily saturated compounds in which the 16 and 18 carbon moieties account for 43 and 28% of the total. This distribution is similar to that observed with the free fatty aldehydes isolated from other bovine hearts where hexadecanal and octadecanal accounted for 63 and 23% of the total aldehydes (4) and is in marked contrast to the cholesteryl alkyl ethers of bovine cardiac muscle where cholesteryl hexadecyl ether accounted for 90% of the total compounds of this type (8). This would suggest that the fatty chain of the cholesteryl alk-1-enyl ether is not derived from the same source as that of the cholesteryl alkyl ether.

Since hexadecanal and octadecanal are the principal fatty chains observed in the cholesteryl alk-1-enyl ethers as well as in the free fatty aldehydes from bovine cardiac muscle, it seems that free fatty aldehydes may play a role in the biosynthesis of cholesteryl alk-1-enyl ethers. Similar suggestions have been made with respect to the biosynthesis of glycerol alk-1-enyl ethers (18, 19).

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