Isolation and analysis of free fatty aldehydes from rat, dog, and bovine heart muscle

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ABSTRACT Procedures are described for the isolation of free fatty aldehydes from total lipid extracts of rat, dog, and bovine heart muscle. These aldehydes did not arise from hydrolysis of the naturally occurring alkenyl ethers in either the extraction or isolation procedures.

The free fatty aldehydes were present in heart muscle in amounts between 0.27 and 0.56 μmole per 100 mg of lipid.

Hexadecanal and octadecanal are the principal free fatty aldehydes present, except in dog heart muscle where an unidentified, unusual aldehyde constitutes 20% of the mixture.

KEY WORDS free fatty aldehydes · rat · dog · bovine · heart muscle · thin-layer chromatography · *p*-nitrophenylhydrazones · dimethyl acetals · gas–liquid chromatography · silicic acid column chromatography

THE EXISTENCE of free fatty aldehydes as naturally occurring components of mammalian lipids has not been demonstrated, although the following observations suggest their existence.

Acylated alkenyl α-glycerol ethers are naturally occurring aldehydogenic lipids in mammalian tissues (1, 2). Recently an enzyme system capable of hydrolyzing 1-alkenyl glycerol 3-phosphorylcholine to yield a fatty aldehyde and glycerol phosphorylcholine has been described in rat liver (3). In *Clostridium butyricum*, palmitaldehyde has been demonstrated as a precursor of the fatty chain of the alkenyl ether as well as the esterified fatty acids of the phosphatides (4). During the biosynthesis of sphingosine by brain tissue, palmitoyl CoA is reduced by NADPH to palmitaldehyde, which is then incorporated into dihydrosphingosine (5). A similar observation has been made in experiments with human subjects. Here an increase in the C_{18} monoenes present in the alkenyl ethers of the erythrocyte plasmalogens was observed after the feeding of large amounts of triolein (6).

In view of these observations, we postulated the natural occurrence of free fatty aldehydes. The information presented in this paper establishes the presence of these lipids in mammalian heart muscle. A preliminary communication relating to the early phases of the work has appeared (7).

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NADH, reduced nicotine adenine dinucleotide; NADPH, reduced nicotine adenine dinucleotide phosphate.

EXPERIMENTAL PROCEDURES

Extraction of Lipids

Dog, rat, and bovine heart muscle was used. The chambers of the heart were opened immediately after removal from the animal and rinsed with 0.9% NaCl. Pericardial fat was rapidly removed in the cold and the tissue homogenized in a Waring Blendor with chloroform–methanol 2:1 (8). Rat and dog heart lipid was extracted immediately after the death of the animal. Bovine heart, obtained from a slaughter house, was cooled in ice during transit and the lipid extraction was completed within 2 hr after the death of the animal. After removal of nonlipid material the lipid extracts were concentrated under vacuum on a rotary evaporator at 33°C, blanketed with nitrogen, taken up in 25 ml of redistilled reagent grade chloroform, and stored at 4°C. Duplicate aliquots of the lipid extract were dried under nitrogen and weighed to determine the total lipid weight.

TLC

The method of preparing neutral thin-layer plates has been previously described (9). Free fatty aldehydes were separated from the other lipid classes by means of

hexane–chloroform–methanol 73.5:25:1.5. Lipid extracts were applied in a streak 2.0 cm from the bottom of the plate. Mixed reference standards were also applied. After the plates were developed, they were sprayed with 0.05% Rhodamine G in 80% methanol. The section with the same mobility as octadecanal was scraped off and eluted with chloroform.

The recovery of fatty aldehydes was assessed by applying 0.07–1.26 μmoles of standard octadecanal to thin-layer plates, developing them with hexane–chloroform–methanol, eluting with chloroform, and quantifying the recovered aldehydes as *p*-nitrophenylhydrazones. Under these conditions the recovery of free fatty aldehyde was 100.6 ± 2.4% SD (n = 10). In some instances tissue free fatty aldehydes were obtained after TLC in two different solvent systems: (*a*) hexane–chloroform–methanol, after which the fatty aldehydes were eluted with chloroform and purified by (*b*) hexane–isobutanol–methanol 100:3:3. The recovery of 1 μmole of standard octadecanal was 92.3 ± 3.4% SD (n = 10).

Column Chromatography

We investigated the separation of fatty aldehydes from other neutral lipids on silicic acid columns in the hope of obtaining a method to prepare large amounts of the aldehydes for IR and nuclear magnetic resonance spectroscopy. A standard mixture containing 50 mg each of cholesteryl stearate, tristearin, octadecanal, and cholesterol was applied to a column containing 18 g of silicic acid and the development begun with 1% ethyl ether in *n*-heptane (10). The eluate was collected in 15-ml fractions. The samples were concentrated and the residues chromatographed on thin-layer plates of Silica Gel G. Fatty aldehydes were eluted in fractions 7–14 together with the cholesteryl esters, but could be separated from the latter by TLC.

Bovine heart neutral lipids were obtained by flocculating the phospholipids with acetone and $MgCl_2$ (11) until less than 1 μg of P was detectable in a neutral lipid sample of 235 mg (12). A total of 12.5 g of this neutral lipid was separated on silicic acid columns and the free fatty aldehydes were isolated. Prior to GLC, IR, and nuclear magnetic resonance spectroscopy, these aldehydes were purified by TLC using the hexane–chloroform–methanol system described above.

GLC

The instrument was an F & M model 400 (F & M Scientific Corp., Avondale, Pa.), with a hydrogen flame ionization detector. Two U-shaped borosilicate glass columns containing 15% ethylene glycol adipate polyester and 16% Apiezon M on alkali-washed, silanized 60–80 mesh Gas-Chrom S were used (13, 14). Analyses were run at 197°C on Apiezon M and at 165°C on the ethylene glycol adipate column. Argon was used as the carrier gas at a flow rate of 112 ml/min with an inlet pressure of 40 psi.

Dimethyl acetal derivatives of both the standard and tissue free fatty aldehydes were prepared by refluxing the aldehydes with anhydrous methanolic HCl (15). The dimethyl acetals were obtained pure by TLC in hexane–chloroform 75:25. Breakdown of dimethyl acetals during GLC has been reported by others (4, 15) but was not observed with either column when standard mixtures were chromatographed.

The retention times of the standard and sample dimethyl acetals were calculated relative to those of the dimethyl acetal of octadecanal. Dimethyl acetals of the free fatty aldehydes were identified by comparison with standards run under identical conditions.

Peak areas were calculated as the product of the peak height and the width at half peak height, and percentage distributions are given in terms of peak areas.

Other Analytical Procedures

Free fatty aldehydes were quantified as the *p*-nitrophenylhydrazones (16). The IR spectra of the free fatty aldehydes were obtained on a Beckman IR 8 instrument (Beckman Instruments, Inc., Fullerton, Calif.) and the nuclear magnetic resonance spectrum on a Model A-60 spectrometer (Varian Associates, Palo Alto, Calif.). Both spectra were obtained with carbon tetrachloride as the solvent.

Radioactivity Determination. The radioactivity of ^{14}C-labeled lipids was determined with the aid of a Packard Tri-Carb liquid scintillation spectrometer, model 4322, with toluene containing 0.4% diphenyloxazole and 0.01% *p*-bis[2-(5-phenyloxazolyl)]-benzene as the scintillator fluid. All samples were counted either for 20 min or for a total of 20,000 counts.

Materials

All solvents were reagent grade and, with the exception of methanol, were redistilled before use. The solid support and liquid phases used for GLC were obtained from the Applied Science Laboratories, Inc., State College, Pa. The fatty aldehyde standards were obtained from the Aldrich Chemical Company, Milwaukee, Wis. Acetate-1-^{14}C was purchased from the New England Nuclear Corp., Boston, Mass. Mallinckrodt silicic acid, 100 mesh, was used in the column chromatographic procedures. Silica Gel G was obtained from the Applied Science Laboratories.

RESULTS

Separation and Identification of Free Fatty Aldehydes

In a previous paper the separation of standard octadecanal from cholesteryl stearate, tristearin, free choles-

terol, and palmitic acid by TLC was demonstrated (7). Fig. 1 shows that by use of the same solvent (hexane–chloroform–methanol 73.5:25:1.5) octadecanal can be separated readily from diolein, coenzyme Q, retinol, and retinol palmitate. This is important because retinol interferes with the *p*-nitrophenylhydrazone determination (17). Fig. 2 shows that free fatty aldehydes can also be separated from cholesterol, cholesteryl stearate, and tristearin with the developing solvent isobutanol–hexane–methanol 3:100:3. Hexane–chloroform–methanol was the solvent system we routinely used for TLC since in this system the mobility of many standard compounds was known.

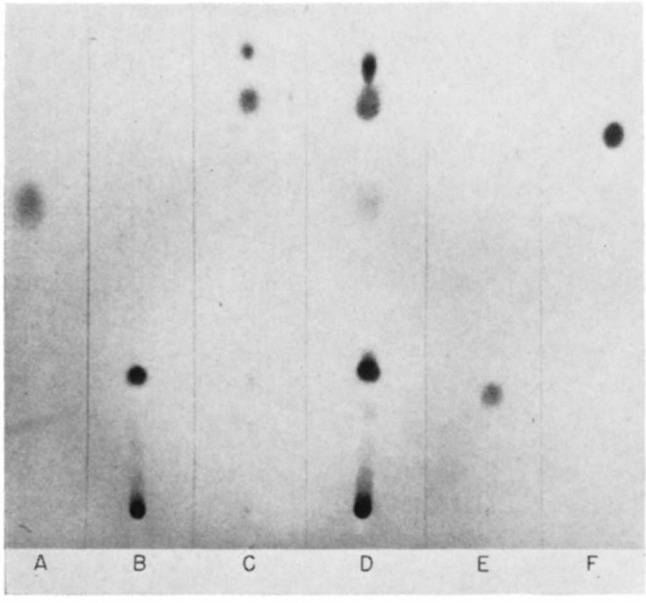

FIG. 1. Thin-layer chromatogram of standards on Silica Gel G. Solvent, hexane–chloroform–methanol 73.5:25:1.5. Indicator, iodine vapor. A, octadecanal; B, retinol; C, retinol palmitate; D, mixed standards; E, diolein; F, coenzyme Q.

As shown in Fig. 3, bovine heart muscle lipid contains a component with the same mobility in hexane–chloroform–methanol as octadecanal. After elution from the plate, this lipid component had the same thin-layer chromatographic mobility in isobutanol–hexane–methanol as octadecanal.

IR and Nuclear Magnetic Resonance Spectra

In Fig. 4 spectrum B represents the dimethyl acetal derivative of the purified free fatty aldehydes of bovine heart. This spectrum is identical with that of hexadecanal dimethyl acetal, spectrum A, with a strong absorption peak characteristic of the acetal structure at 1114 cm^{-1}. The absorption bands at 2926 and 2853 cm^{-1} are those characteristic of the hydrocarbon structure of the molecule, as is the peak at 1456 cm^{-1} (18). In essence the

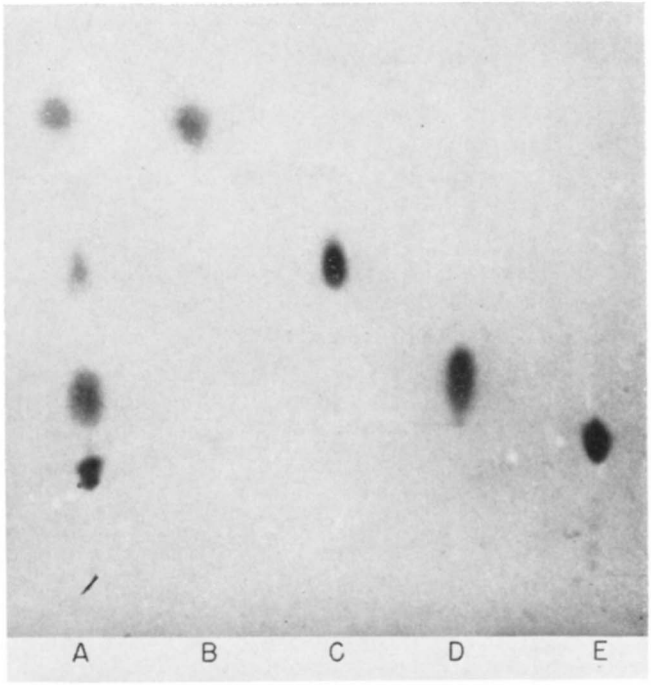

FIG. 2. Thin-layer chromatogram of standards on Silica Gel G. Solvent, hexane–isobutanol–methanol 100:3:3. Indicator, iodine vapor. A, mixed standards; B, cholesteryl stearate; C, tristearin; D, octadecanal; E, cholesterol.

spectrum is that expected for a mixture of aliphatic dimethyl acetals, the majority of which are saturated. This is borne out by the nuclear magnetic resonance and gas chromatographic data.

Fig. 5 is the spectrum of the free fatty aldehydes from bovine heart neutral lipids. All chemical shift values are reported as τ values in parts per million with tetramethylsilane as an internal standard and CCl_4 as the solvent. The characteristic triplet of the aldehyde proton is observed at $\tau = 0.27$. A value of $\tau = 0.26$ has been reported for *n*-butyraldehyde (19). Confirmation of the hydrocarbon chain is provided by the methyl group ($\tau = 9.12$), the methylenes ($\tau = 8.74$), and a weak signal ($\tau = 7.64$) characteristic of the protons on a carbon alpha to the carbonyl function.

Gas–Liquid Chromatography

The gas chromatographic tracing of the dimethyl acetals prepared from the bovine heart free fatty aldehydes is presented in Fig. 6. A series of components is present, of which hexadecanal and octadecanal are the principal components. The dimethyl acetals were identified by comparing their retention times, relative to that of the dimethyl acetal of octadecanal, with standard values obtained on the same column and with values in the literature (20). The relative retention times for standard dimethyl acetals on an Apiezon M column are presented in Table 1.

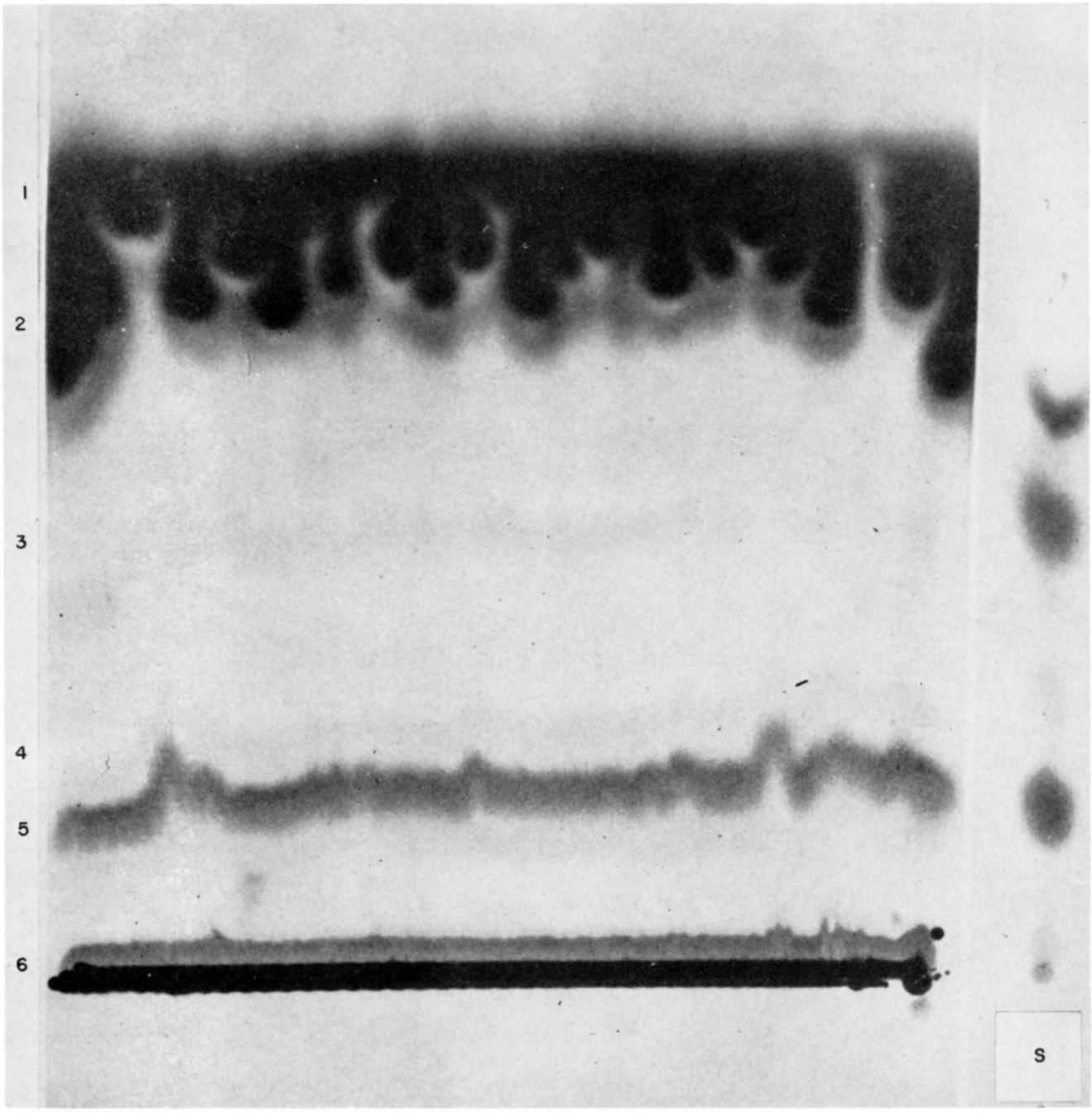

FIG. 3. Thin-layer chromatogram of bovine heart lipids and standards on Silica Gel G. Solvent, hexane–chloroform–methanol 73.5:25:1.5. Indicator, iodine vapor. Lane *S* contains mixed standards: from origin to front, palmitic acid, cholesterol, diolein, octadecanal, and coenzyme Q (the small spot at the origin is an artifact and should be ignored). Positions of the lipid classes: 1, cholesteryl esters and triglycerides; 2, coenzyme Q; 3, free fatty aldehydes; 4, diglycerides; 5, cholesterol; 6, free fatty acids and phospholipids.

Table 2 gives the percentage distribution of the dimethyl acetals of the free fatty aldehydes isolated from bovine, rat, and dog hearts.

Stability of Alkenyl Glycerol 1-Ethers

Since alkenyl glycerol 1-ethers are known to give rise to fatty aldehydes on acid hydrolysis, the stability of the naturally occurring alkenyl ethers during the extraction and isolation procedures was evaluated in several ways. It is pointed out that in all our procedures an acidic environment was avoided.

Stability in Tissue Post Mortem. Since extraction of the lipids could not be completed until about 2 hr after the death of the animal, the possibility that the free fatty

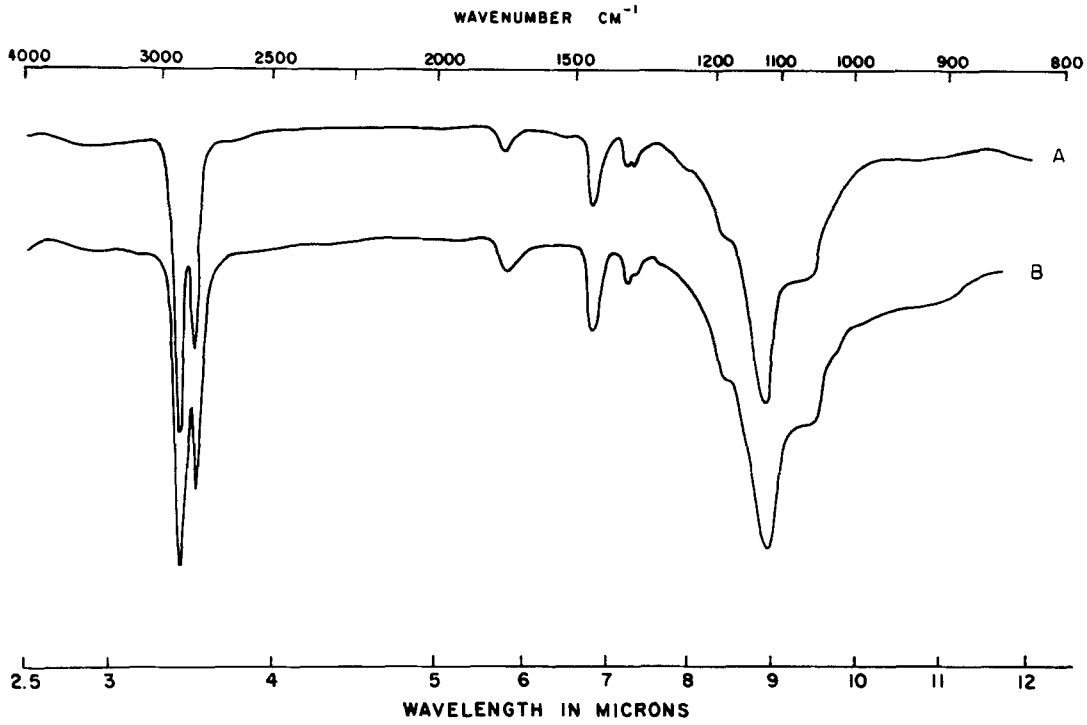

FIG. 4. IR spectra of (*A*) hexadecanal dimethyl acetal and (*B*) dimethyl acetals of free fatty aldehyde from bovine heart lipids. Solution of 10% lipid in CCl_4 in a NaCl cell.

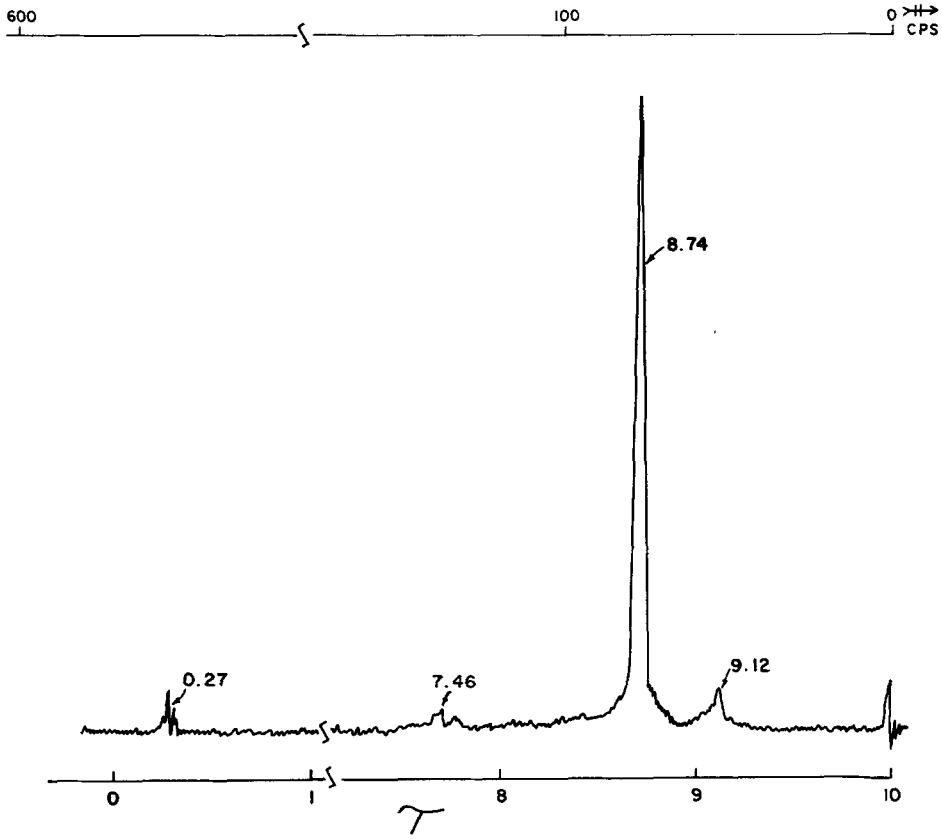

FIG. 5. Nuclear magnetic resonance spectrum of free fatty aldehydes from bovine heart, in CCl_4 solution. Values are in parts per million with respect to tetramethylsilane as an internal standard.

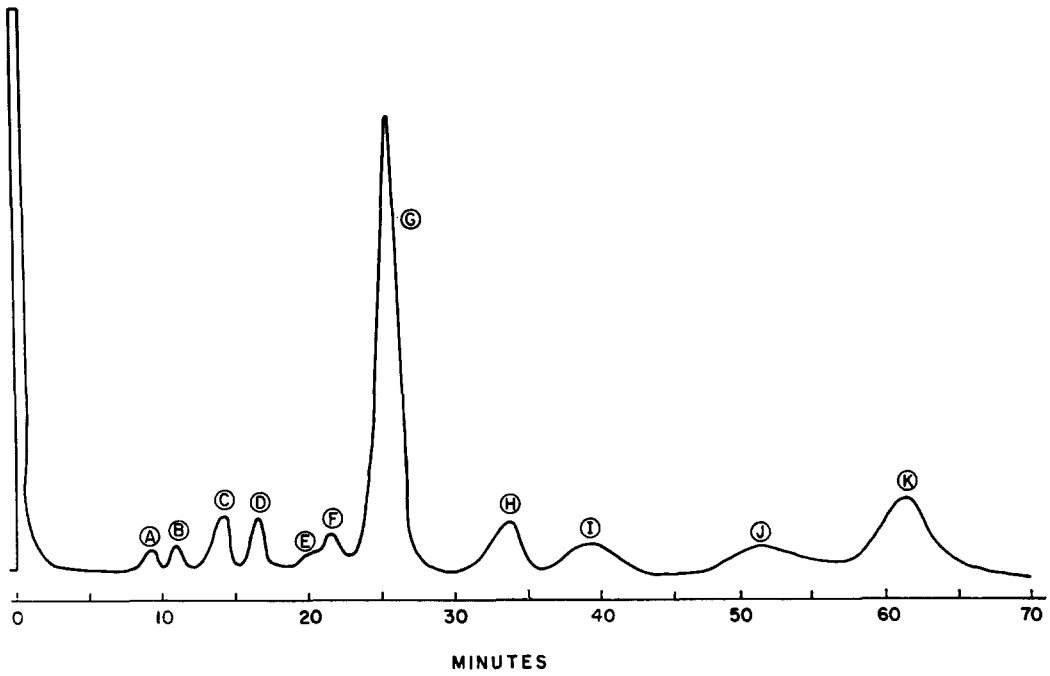

FIG. 6. Gas–liquid chromatogram: aldehyde dimethyl acetals from bovine heart lipids on Apiezon M. Chain-lengths and double bonds: *A*, 13:0: *B*, 14:0; *C*, 15:0 (iso or anteiso); *D*, 15:0; *E*, unknown; *F*, 16:1; *G*, 16:0; *H*, 17:0 br; *I*, unknown; *J*, 18:1; *K*, 18:0.

TABLE 1 RELATIVE RETENTION TIMES OF DIMETHYL ACETALS ON APIEZON M

Shorthand Designation*	Natural Mixture	Standards	Literature (18)
10:0	0.046	0.045	
11:0	0.069		
12:0	0.090	0.090	
13:0	0.134		
14:0	0.177	0.179	0.180
15:0 br	0.221		0.239
15:0	0.271		0.272
Unident.	0.309		
16:1^{9} *cis*	0.362		0.361
16:0	0.420	0.420	0.424
17:0 br	0.555		0.567
17:0	0.647		0.646
18:2^{9,12} *cis*	0.780		0.782
18:1^{9} *cis*	0.869		0.847
18:0	1.020	1.000	1.000

All retention times are relative to that the dimethyl acetal of standard octadecanal. Dimethyl acetals designated as 11:0 and 13:0 were identified from a plot of the relative retention time of standard dimethyl acetals versus their molecular weight.

* For the corresponding aldehyde. No. of carbons: No. of double bonds, br = branched.

TABLE 2 PERCENTAGE DISTRIBUTION OF THE FREE FATTY ALDEHYDES OF HEART MUSCLE

	Rat	Bovine	Dog
10:0	tr.		4.9
11:0	tr.		tr.
12:0	tr.		tr.
13:0	3.2	tr.	1.0
14:0	3.8	tr.	1.2
15:0 br		3.4	2.6
15:0	tr.	6.1	10.5
Unident.*		tr.	20.3
16:1	9.8	tr.	
16:0	45.7	62.6	19.8
17:0 br	tr.	5.0	9.9
17:0			10.6
18:2			6.7
18:1	21.0	tr.	tr.
18:0	16.4	22.9	10.9

tr., less than 1% of the total.

* Relative retention time, 0.309 on Apiezon M.

aldehydes arose in that interval was examined. Two dog hearts were obtained at the time of death and immediately rinsed in isotonic saline; a slice of muscle was removed, and the lipid was extracted. This procedure was completed within 5 min of the time of death. The remaining muscle was cut into several portions which were weighed and stored in ice. From different portions of tissue, taken at 30-min intervals over a period of 2 hr after the death of the animal, the lipid was extracted (8). Each lipid extract was analyzed for free fatty aldehydes and for total fatty aldehydes released by acid hydrolysis (16). From Table 3 it can be seen that the free fatty aldehydes represent a virtually constant fraction of the total fatty aldehydes in each heart. If the alkenyl ethers were undergoing hydrolysis post mortem, the ratio of free fatty aldehydes would increase with time.

Stability during Extraction and Analysis. Radiolabeled alkenyl ethers were prepared biosynthetically. Acetate-

TABLE 3 STABILITY OF DOG HEART ALKENYL ETHERS PRIOR TO EXTRACTION OF TISSUE LIPIDS

Time after Death	Tissue Weight	Total Lipid	Fatty Aldehydes		% Free
			Free	Total	
min	*g*	*mg*	*μmoles*		
5	21.045	536	6.99	146	4.8
30	20.749	605	7.66	156	4.9
60	14.316	392	4.33	104	4.2
90	21.352	468	6.33	136	4.6
120	21.409	579	5.66	144	4.0
5	19.260	846	5.33	140	3.8
30	18.200	940	4.33	123	3.5
60	19.156	705	5.00	164	3.0
90	19.271	868	5.37	158	3.4
120	20.320	751	6.33	158	4.0

1-^{14}C, 20 mc/100 g of body weight, was injected into two adult male rats. The injections were continued for 4 days and the animals were killed on the 5th day. The internal organs, consisting of heart, brain, kidney, and epididymal fat pads, were removed from one animal and the gastrocnemius and gluteus maximus muscles from the other. The tissue lipids were extracted as before and the lipid extract was concentrated under reduced pressure. The free fatty aldehydes were separated from the other lipid classes by TLC as above and rechromatographed in hexane–isobutanol–methanol 100:3:3. The aldehydes were eluted, quantified as *p*-nitrophenylhydrazones (16), and counted in a liquid scintillation spectrometer.

To determine the radioactivity of the bound aldehydes, we took an aliquot from both lipid extracts, after the removal of free fatty aldehydes by TLC, and heated each lipid residue with 90% acetic acid at 37°C for 17 hr (21). The fatty aldehydes released by acid hydrolysis were separated by TLC, quantified as *p*-nitrophenylhydrazones, and counted.

The results of this experiment are given in Table 4. It can be seen that the specific activity of the free fatty aldehydes in skeletal muscle is some 44 times and that in the internal organs 32 times greater than that of bound aldehydes. Thus, in both instances it would have been impossible for the free fatty aldehydes to have arisen from hydrolysis of alkenyl ethers during extraction or chromatography.

Further proof that alkenyl ethers do not give rise to free fatty aldehydes during the extraction or isolation procedures was obtained as follows: an aliquot of the radiolabeled lipid extract from the internal organs, from which the free fatty aldehydes had been removed by TLC, was added to the homogenizing flask prior to extraction of the lipids from 14.08 g of bovine muscle. The added ^{14}C-labeled lipid contained a total of 11.87 μmoles of alkenyl ethers (quantified as the *p*-nitrophenylhydrazones) with a specific activity of 33 dpm/μmole. The total radioactivity added, contained in both alkenyl ethers and other

TABLE 4 INCORPORATION OF INTRAPERITONEALLY INJECTED ACETATE-1-^{14}C INTO FREE AND BOUND FATTY ALDEHYDES OF RAT TISSUES

Tissue	Amount of Aldehyde		Specific Activity of Aldehydes	
	Free	Bound	Free	Bound
	μmoles/100 mg lipid		*dpm/μmole*	
Skeletal muscle	0.48	5.80	1354	31
Internal organs	1.46	19.79	1049	33

fatty chains, was 87,840 dpm. A total of 545 mg of lipid was extracted from the tissue and applied to a silicic acid column, and free fatty aldehydes were eluted with 1% diethyl ether in *n*-heptane. The remaining lipids were eluted with 400 ml of diethyl ether followed by 400 ml of methanol (10). When the fractions were combined, a total of 86,590 dpm was observed, which indicates that recovery, based on radioactivity, was greater than 98%. The free fatty aldehydes were isolated by TLC and quantified and their radioactivity was determined. A total of 0.97 μmole of free fatty aldehyde was obtained but no radioactivity was detected in this fraction.

Stability on Storage. Samples of dog heart lipid that were used to obtain the data in Table 3 and had since been stored in *n*-heptane for 2 months at 4°C were reassayed for free and total fatty aldehydes (16). When the values obtained in the second analysis were expressed as a percentage of those observed initially, an average of 101.6 ± 4% (SD, n = 8) was observed for the free and a value of 100.3 ± 3.1% for the total fatty aldehydes.

Free Fatty Aldehyde Content of Heart Muscle. The reproducibility of the values for free fatty aldehyde was ±2.8% (SD, n = 4) of the mean (0.18 μmole) for aliquots of lipid from dog heart muscle. Values for free and total fatty aldehydes in heart muscle are presented in Table 5. Free fatty aldehydes comprise 1.4–3.5% of the total fatty aldehydes.

TABLE 5 FREE FATTY ALDEHYDE CONTENT OF HEART MUSCLE

	Wet Weight	Total Lipid	Hydrazone Formed		% Free
			Total	Free	
	g	*mg*	*μmoles*		
Rat*	11.18	225	36.20	1.27	3.5
Bovine	18.60	720	40.49	1.05	2.6
Dog	29.36	856	159.17	2.29	1.4

* Pooled tissues from 12 male rats with average body weight of 250 g.

DISCUSSION

Free fatty aldehydes have been demonstrated as a naturally occurring neutral lipid in heart muscle; their presence cannot be attributed to hydrolysis of the

naturally occurring alkenyl ethers either prior to or after extraction of the tissue lipids.

That free fatty aldehydes are synthesized in vivo by mammalian tissues is indicated by the observation that the ^{14}C-labeled free fatty aldehydes were isolated from the internal organs and skeletal muscle of the rat when acetate-1-^{14}C was given intraperitoneally. This conclusion is further supported by the observation (unpublished) that at 1- and 4-hr intervals after an infusion of albumin-bound palmitate-1-^{14}C into two dogs, ^{14}C-labeled free fatty aldehydes could be isolated from the serum. Possibly, a dehydrogenase enzyme that is capable of reducing fatty acids to fatty aldehydes is present. It is known that palmitoyl CoA can be reduced in the presence of NADH to palmitaldehyde by a rat brain supernatant fraction (22) and that acetaldehyde dehydrogenase from *Clostridium butyricum* requires CoA (23). Earlier work with deuterated fat had suggested the ability of the rat to reduce fatty acids to the corresponding fatty aldehydes (24); although these were bound aldehydes, the mechanism may be the same.

An alternative source of free fatty aldehydes is suggested by a more recent study with rat liver (25), in which it was shown that batyl alcohol could be oxidized to glycerol and a fatty acid. During the process a fatty aldehyde could be trapped as the hydrazone.

The principal fatty aldehydes present (Table 2) are hexadecanal and octadecanal and most of the others are saturated (C_{11}–C_{18}). One major unidentified component in dog heart muscle (20.3%, dimethyl acetal eluted between 15:0 and 16:1 from Apiezon M) had a retention time relative to 18:0 of 0.592 on ethylene glycol adipate polyester (hexadecanal 0.497). Retention times of the other dimethyl acetals on ethylene glycol adipate polyester (not shown) confirmed the identification deduced from the Apiezon column.

Although a biochemical role for free fatty aldehydes in mammalian tissues is not apparent, the possibility that these compounds are involved in the synthesis of alkenyl ethers is suggested by the incorporation of palmitaldehyde into alkenyl ethers (4) and by our observation in rat that after injection of acetate-^{14}C the specific activity of the free aldehydes (at least in skeletal muscle) is at least 30 times greater than that of acid-hydrolyzable bound aldehydes.

Bioluminescent bacteria are known to require a fatty aldehyde for the oxidation of reduced flavine mononucleotide and for luminescence (26, 27), a fatty acid being suggested as a product of the process. Whether free fatty aldehydes play a role in the oxidation of reduced flavine mononucleotides in mammalian tissues has never been considered.

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