Microanalysis of cardiolipin in small biopsies including skeletal muscle from patients with mitochondrial disease

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Abstract Cardiolipin is a specific mitochondrial phospholipid that is present in mammalian tissues in low concentration. To measure cardiolipin in small biopsies from patients with mitochondrial disease, we developed a new technique that can detect subnanomolar levels of well-resolved molecular species, the most abundant of which are tetralinoleoyl-cardiolipin (L_4) and trilinoleoyl-oleoyl-cardiolipin (L_3O). To this end, a fluorescence-labeled derivative of cardiolipin (2-[naphthyl-1′-acetyl]-cardiolipin dimethyl ester) was formed and analyzed by high performance liquid chromatography. Cardiolipin was measured in skeletal muscle biopsies from 8 patients with mitochondrial disease and in 17 control subjects. In 5 patients with mitochondrial disease, cardiolipin content was higher than normal (2.4–7.0 vs. 0.4–2.2 nmol/mg protein). In 3 patients with mitochondrial disease, the L_4/L_3O ratio was lower than normal (2–4 vs. 4–6). Cardiolipin was also measured in various rat and dog muscle tissues. The L_4/L_3O ratio was higher in condensed "muscle" type mitochondria (heart ventricle, skeletal muscle, ratios 4–7) than in orthodox "liver" type mitochondria (liver, smooth muscle, heart auricular appendage, H9c2 myoblasts, ratios 0.4–3), suggesting that the L_4/L_3O proportion is important for cristae membrane structure. We concluded that the L_4/L_3O ratio is a tissue-specific variable that may change in the presence of mitochondrial disease. The new method is suitable to measure cardiolipin in muscle biopsies in order to estimate concentration of mitochondria.—Schlame, M., S. Shanske, S. Doty, T. König, T. Sculco, S. DiMauro, and T. J. J. Blanck. **Microanalysis of cardiolipin is small biopsies including skeletal muscle from patients with mitochondrial disease. *J. Lipid Res.* 1999.** 40: **1585–1592.**

Supplementary key words fluorescence • lipid analysis • high performance liquid chromatography • mitochondrial DNA • neuromuscular disease

Cardiolipin is an acidic phospholipid with a unique dimeric structure including four fatty acids (1). It is found in both bacteria and mitochondria. While bacteria express variable amounts of cardiolipin, depending on the stage of growth, mitochondria contain cardiolipin as an essential component of their cristae membrane (2–5). Until recently it was believed that mitochondria are absolutely dependent on cardiolipin. However, a cardiolipin synthase null mutant in *Saccharomyces cerevisiae* was viable (6), probably because phosphatidylglycerol can replace cardiolipin (7). Although the exact biological function of cardiolipin still remains to be defined, its high affinity to various membrane proteins suggests involvement in the intricate structure of the mitochondrial cristae membrane (3–5). Indeed, a mammalian cell line made deficient in cardiolipin showed markedly altered mitochondrial ultrastructure (8).

Cardiolipin was first discovered in beef heart tissue (9). Soon it was recognized that it was not specific to the heart but it was merely more abundant there because of the high concentration of mitochondria in cardiac myocytes. In tissues that contain less mitochondria, it has been notoriously difficult to measure cardiolipin by conventional lipid analytical techniques. This has become a problem because there has been renewed interest in cardiolipin as a potential factor in several pathologies, such as thyroid disease (10–12), adriamycin toxicity (13), free radical-mediated disorders (14), complement activation (15), as well as in aging (12, 16). Some progress in the microdetection of cardiolipin has been made by using the fluorescent dye 10-N-nonyl acridine orange, which displays specificity for cardiolipin upon binding to intact membranes (17).

To date, thyroid dysfunction is the only disease that has been unequivocally linked to changes in cardiolipin content. While the hypothyroid state leads to a decrease of cardiolipin (18), the opposite is true for hyperthyroidism (19), and a direct effect of thyroxin on the biosynthetic enzymes of cardiolipin was reported (10, 20). As thyroxin

Abbreviations: HPLC, high performance liquid chromatography; L_4, tetralinoleoyl-cardiolipin; L_3O, trilinoleoyl-oleoyl-cardiolipin; L_2O_2, dilinoleoyl-dioleoyl-cardiolipin; MELAS, multifocal encephalomalacia, lactic acidosis, and stroke-like episodes.

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is a well-known stimulator of mitochondrial biogenesis, it may be hypothesized that cardiolipin has some role in the formation of mitochondria. It should be noted that mammalian cardiolipin displays an unusual preference for linoleic acid (2–4, 21). As this preference is not shared by other mammalian phospholipids, it can be inferred that the specific acyl pattern of cardiolipin is important for its function in the mitochondria. Therefore, analysis of cardiolipin in pathological tissues ought to measure both cardiolipin content and its pattern of molecular species.

To study cardiolipin in muscle biopsies from children with mitochondrial disease, we developed a novel technique to quantitate cardiolipin in small tissue samples. The method exploits the fact that the acid form of cardiolipin can be methylated yielding an unusually apolar phospholipid, which can be easily separated from normal phospholipids and is very suitable for reversed-phase HPLC. The methylated cardiolipin was labeled with the naphthyl moiety to allow HPLC with fluorescence detection. The chromatography resolved molecular species of cardiolipin.

MATERIALS AND METHODS

Patients

Skeletal muscle biopsies were obtained for diagnostic purposes from adults (age 18–74 years) and children (age 1–12 years). Additional muscle samples were obtained from patients who underwent knee surgery using a protocol approved by the institutional review board of the Hospital for Special Surgery. All patients gave written informed consent. Patients included eight subjects with well-documented mitochondrial disorders: four children (three males, one female, ages 1 to 9 years) with generalized cytochrome oxidase deficiency, two women (ages 18 and 35 years) with MELAS 3243 (A3243G point mutation in the $tRNA^{Leu(UUR)}$ gene of mitochondrial DNA, producing a syndrome characterized by multifocal encephalomalacia, lactic acidosis, and strokelike episodes), one child (female, 1 year) with Leigh syndrome (T8993G mutation of mitochondrial DNA), and one man (age 34 years) with multiple deletions of mitochondrial DNA. The latter case has been reported before (22).

Materials

Muscle tissues were obtained from male Sprague-Dawley rats (skeletal, smooth, and heart muscles) and female beagles (heart muscles). Rat skeletal muscle tissue was obtained from the diaphragm. Rat smooth muscle was taken from the esophagus. The animals were anesthetized with isoflurane in a procedure approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery. Tissues were homogenized in 50 mm Tris-buffered 0.15 m KCl (pH 7.5) using first a Polytron PT300 tissue grinder and then a Potter-Elvehjem homogenizer (100 mg tissue wet weight per ml buffer). The H9c2 myoblast cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, harvested by trypsin digestion, collected by centrifugation, and resuspended in buffered saline prior to lipid extraction. Tetrastearoyl-cardiolipin was produced by hydrogenation of commercial bovine heart cardiolipin (Sigma, St. Louis, MO). The commercial cardiolipin (2.5 mg) was dried under a stream of nitrogen and redissolved in 1.0 ml acetonitrile–ethanol 1:1 (by volume). Platinium(IV) oxide (25 mg) was added and the suspension was bubbled with hydrogen gas for 30 min. The catalyst was removed by centrifugation and filtration, the clear solution was dried under nitrogen and redissolved in ethanol–chloroform 2:1 (by volume). Supelclean solid-phase extraction tubes were obtained from Supelco (Bellefonte, PA) and the C18-Hypersil HPLC column was from Sigma (St. Louis, MO). Naphthylacetic anhydride was obtained from Aldrich (Milwaukee, WI). All other chemicals were of analytical grade.

Quantitative analysis of cardiolipin

Lipids were extracted from muscle tissue (50–100 mg wet weight per sample) or cell homogenates ($2–4 \times 10^6$ cells per sample) according to Bligh and Dyer (23). Tetrastearoyl-cardiolipin (3.6 nmol) was added as an internal standard at the initial step of lipid extraction. After extraction, lipids were dried under nitrogen and acidified by the following method. Ice-cold methanol (2 ml), chloroform (1 ml), and 0.1 m HCl (1 ml) were added, the sample was vortexed and incubated on ice for 5 min. Phase separation was achieved by addition of 1 ml of chloroform and 1 ml of 0.1 m HCl and the chloroform-phase was recovered, followed by re-extraction with 2 ml of chloroform. The chloroform extract was dried under nitrogen and the lipids were treated with diazomethane. To this end, diazomethane was released from 1 g of N-methyl-N-nitroso-*p*-toluenesulfonamide by addition of 2 ml of ethanol and 0.3 ml of 16 m KOH, and the gas was trapped in 16 ml of ice-cold chloroform. One milliliter of the chloroform solution, containing diazomethane, was added to the dry lipid residue and incubated on ice for 15 min. After that, the solvent was evaporated and the methylated lipids were redissolved in 0.2 ml of chloroform. This solution was purified by solid-phase extraction on Supelclean LC-Si tubes (100 mg silica per tube) equilibrated with diethylether–ethanol 9:1 (by volume). After loading the sample, the column was eluted with 2 ml diethylether–ethanol 9:1. The eluate was dried under nitrogen and then redissolved in a reagent consisting of 25 μmol 1-naphthylacetic anhydride and 25 μmol N,N-dimethyl-4-aminopyridine dissolved in 250 μl anhydrous pyridine. This mixture was incubated for 2 h at 40°C. Then, 6 ml of n-hexane was added, the sample was spun to sediment non-soluble material, and the supernatant was further purified by extraction with water. The hexane-phase was treated with anhydrous sodium sulfate to remove traces of water and then evaporated to dryness. The dry residue was dissolved in 1 ml of n-hexane–diethylether 1:1 (by volume) and subjected to another solid-phase extraction step. Supelclean LC-Si tubes (100 mg silica) were equilibrated in n-hexane–diethylether 1:1, the sample was loaded onto the column and eluted with another 2 ml of n-hexane–diethylether 1:1. Next, the column was eluted with 2 ml of diethylether–ethanol 95:5 (by volume) and this eluate was collected, dried, and redissolved in 0.1 ml of n-hexane–ethanol 1:1 (by volume). Twenty microliters of this solution was separated by HPLC using a C18-Hypersil (5 μm) column (150 × 3.2 mm). A solvent gradient was run from acetonitrile–2-propanol 8:2 (by volume) to acetonitrile–2-propanol 5:5 in 30 min. The HPLC was a Shimadzu system consisting of two LC-10AT solvent delivery systems (total flow rate 1.0 ml/min), a Rheodyne manual 20-μl loop injector, and RF-10AXL fluorescence detector (excitation wavelength 280 nm, emission wavelength 360 nm, gain = 2, sensitivity = 2). Data were collected and processed by the Shimadzu CLASS VP software running on a desktop computer. For peak identification, fractions were collected and processed for measurement of the fatty acid profile.

Other methods

For electron microscopy, muscle samples were dissected into 2–4 mm cubes and placed into cold fixative for 12–18 h. The fixative consisted of 2% paraformaldehyde plus 0.5% glutaral-

dehyde in 0.5 m cacolydate buffer (pH 7.2). Tissue samples were dehydrated through a graded alcohol series and embedded in Spurr's resin. Thin sections were collected and stained with Reynolds lead stain and alcoholic uranyl acetate. Micrographs were taken at 13,000 original amplification for all samples, using a Philips CM-12 transmission electron microscope.

Protein concentration was determined according to Lowry et al. (24). Ultraviolet absorbance spectra were recorded with a Beckman DU-7400 spectrophotometer. Fluorescence spectra were recorded with a SLM-Aminco fluorescence photometer. Fatty acids were measured by gas chromatographic analysis of their methyl esters (25), using a Supelcowax 10 capillary column (Supelco, Bellefonte, PA) installed in a GC-17A Shimadzu gas chromatograph. A temperature gradient was run from 180 to 240°C at 5°C/min. Citrate synthase activity was determined spectrophotometrically as described by Srere (26). One enzyme unit was defined as 1 μmol citrate formed per minute. Phospholipid concentration was determined by colorometric measurement of phosphate liberated by ashing (27). For statistical presentation, means are given with standard deviation. Patient groups were compared by the Mann-Whitney rank sum test.

RESULTS

Microanalysis of cardiolipin

We syntheszied a new chemical derivative of cardiolipin in order to develop a sensitive and quantitative assay based on HPLC with fluorescence detection. To this end, a naphthyl-1′-acetyl moiety was linked to the only free hydroxyl group of dimethylcardiolipin. The product showed the characteristic absorbance pattern of the naphthyl group as well as a fluorescence signal centered around 367 nm when excited at 280 nm (**Fig. 1**).

For the purpose of quantitative determination of cardiolipin in biological extracts, the chemical derivatization was combined with a step-by-step purification designed to eliminate other lipids and to isolate the cardiolipin derivative. First, derivatization with diazomethane was performed on the whole lipid mixture. This reaction converted only acidic phospholipids to their methyl esters. In the subsequent solid phase extraction, phospholipid methyl esters

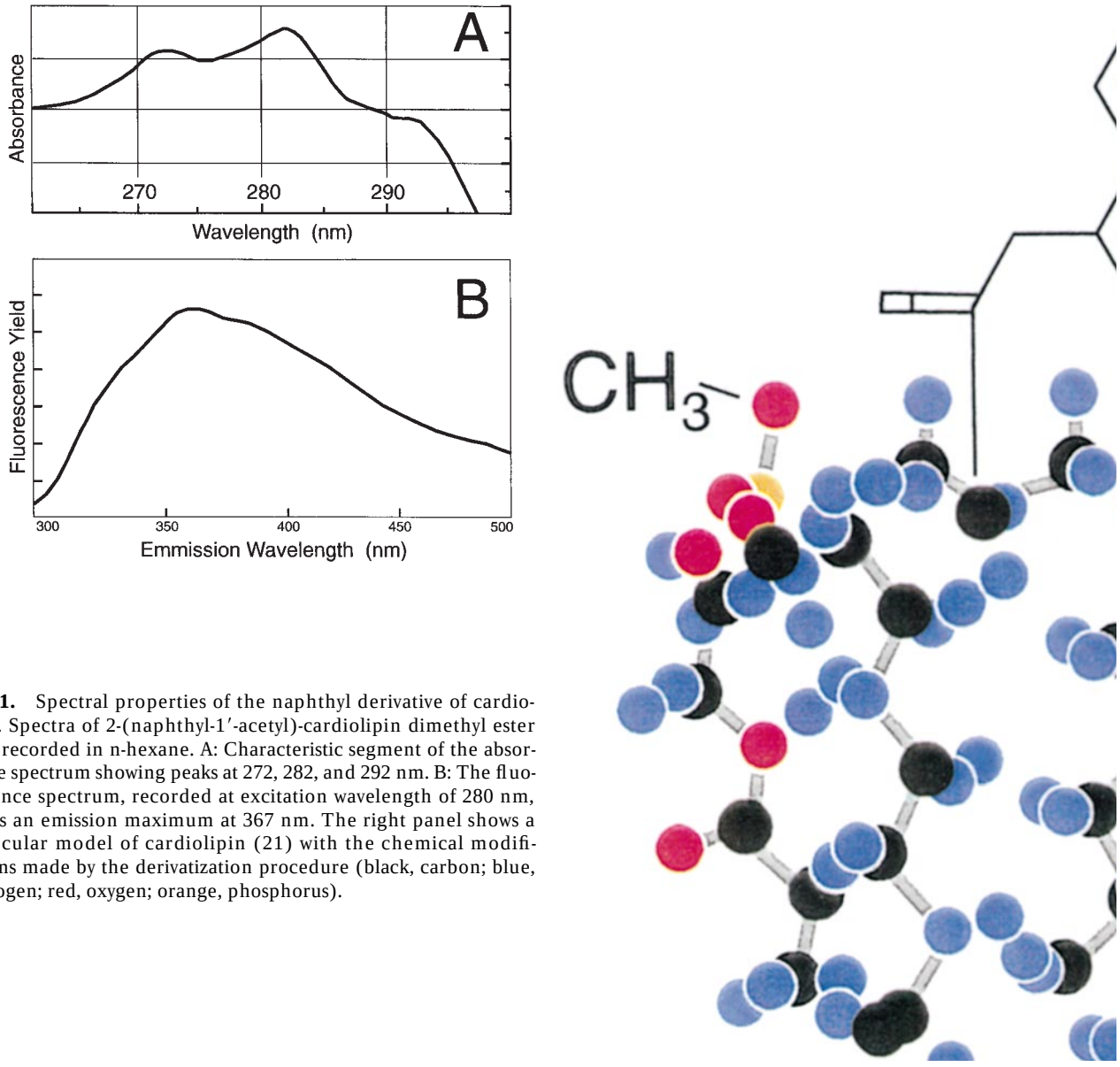


Fig. 1. Spectral properties of the naphthyl derivative of cardiolipin. Spectra of 2-(naphthyl-1′-acetyl)-cardiolipin dimethyl ester were recorded in n-hexane. A: Characteristic segment of the absorbance spectrum showing peaks at 272, 282, and 292 nm. B: The fluorescence spectrum, recorded at excitation wavelength of 280 nm, shows an emission maximum at 367 nm. The right panel shows a molecular model of cardiolipin (21) with the chemical modifications made by the derivatization procedure (black, carbon; blue, hydrogen; red, oxygen; orange, phosphorus).

were sufficiently non-polar to be eluted from silica by diethylether–ethanol 9:1. Thus, lipid separation after methylation was associated with considerable purification. Then, the naphthyl-1′-acetyl group was attached and the sample was further purified to make it suitable for analytical HPLC. The gradual purification of derivatized cardiolipin was documented by thin-layer chromatography (data not shown).

Analytical HPLC of 2-(naphthyl-1′-acetyl-)cardiolipin dimethyl ester, derived from human skeletal muscle, is shown in **Fig. 2**. Four cardiolipin peaks were obtained, representing four different molecular species. The fifth peak was the internal standard, a synthetic cardiolipin species that does not occur in nature. The time-integral of the fluorescence peak was proportional to the amount of cardiolipin (**Fig. 3A**), so cardiolipin concentrations could be calculated in reference to the internal standard. The retention time of molecular species is expected to increase with increasing length of the acyl chains and decrease with increasing degree of unsaturation. Accordingly, we found the logarithmic retention time to be inversely proportional to the total number of double bonds in all-C_{18} species of cardiolipin (Fig. 3B).

Cardiolipin from animal muscle tissues

Cardiolipin content was measured in various muscle tissues from rat and dog, using the fluorescence–HPLC method described above (**Fig. 4**). The content was highest in heart muscle, much lower in skeletal muscle, and lowest in smooth muscle. In heart, the ventricle contained significantly more cardiolipin than the auricular appendage. The cardiolipin content of cultured H9c2 myoblasts was also measured. The cell culture contained 2.9 ± 0.8 nmol cardiolipin per milligram protein (n = 3), about as much as skeletal muscle tissue. This corresponded to 2.2 ± 0.6 fmol of cardiolipin per single cell.

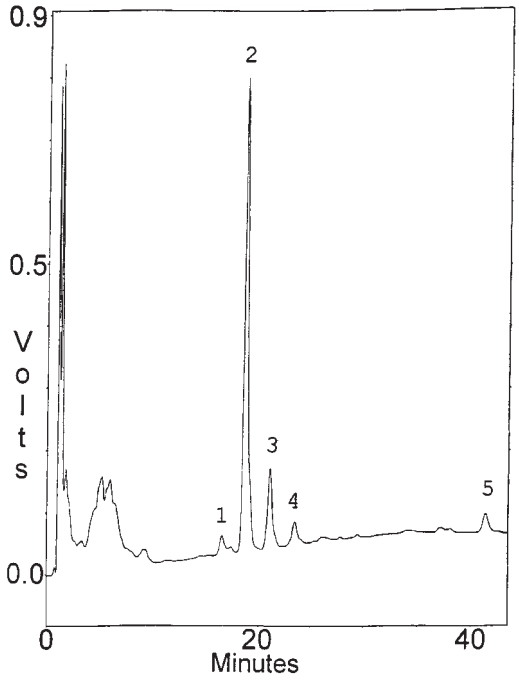


Fig. 2. HPLC of cardiolipin from human skeletal muscle. Cardiolipin was extracted from a skeletal muscle biopsy of a child with cytochrome oxidase deficiency. The HPLC chromatogram shows the fluorescence signal, recorded on a voltage scale, versus the retention time. Peaks represent molecular species of muscle cardiolipin: trilinoleoyl-linolenoyl-cardiolipin (peak 1), tetralinoleoyl-cardiolipin (L_4, peak 2), trilinoleoyl-oleoyl-cardiolipin (L_3O, peak 3), and dilinoleoyl-dioleoyl-cardiolipin (L_2O_2, peak 4). The internal standard (tetrastearoyl-cardiolipin) is in peak 5.

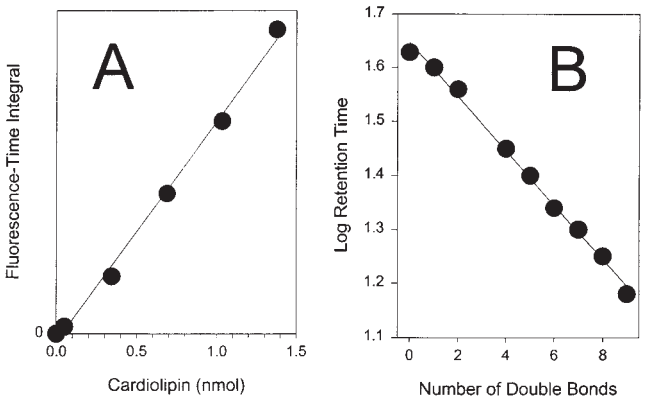


Fig. 3. Quantitative and qualitative analysis of cardiolipin. A: Commercial cardiolipin (bovine heart) was subjected to the analytical procedure. The committed amount of cardiolipin was plotted against the total peak area in the fluorescence chromatogram. B: Retention time of molecular species of partially hydrogenated cardiolipin from bovine heart. All species contain four C_{18}-chains and a variable number of double bonds.

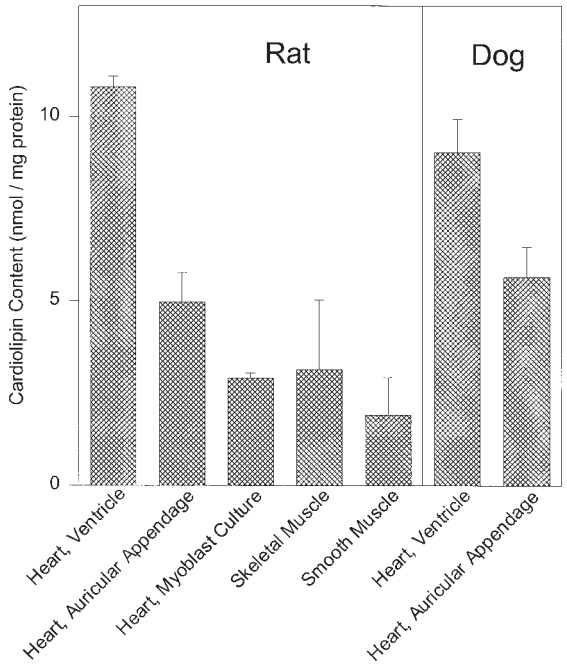
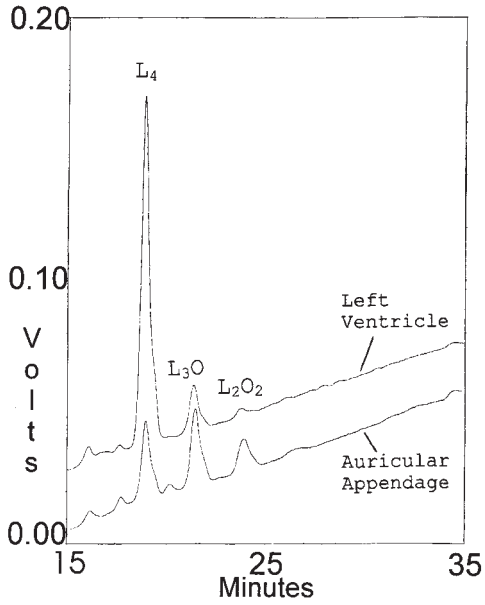


Fig. 4. Cardiolipin content of various muscle tissues. Lipid extracts from muscle tissues were derivatized, purified, and analyzed by HPLC with fluorescence detection. For each tissue, data were obtained from three independent samples.



Fig, 5. HPLC of cardiolipin from rat heart. The figure shows the chromatogram of cardiolipin extracted from the left ventricle and from the auricular appendages. L_4: tetralinoleoyl-cardiolipin, L_3O: trilinoleoyl-oleoyl-cardiolipin, L_2O_2: dilinoleoyl-dioleoyl-cardiolipin.

The two most abundant cardiolipin species in muscle tissues were L_4 and L_3O, together accounting for more than 80% of total cardiolipin. In rat heart, there was a remarkable difference between cardiolipins extracted from ventricle and auricular appendage (**Fig. 5**). Whereas ventricle cardiolipin was predominantly L_4, the appendage contained L_4 and L_3O in about equal amounts. We found the L_4/L_3O ratio to be a characteristic feature of each type of muscle (**Fig. 6**). In rat, the ratio varied from values higher than 5 to values lower than 0.5. Muscle tissues with high L_4/L_3O ratio, such as heart ventricle and skeletal muscle, contained mitochondria with a very regular arrangement of stacked cristae membranes (**Fig. 7, A** and **C**). This is a well-known characteristic of heart and skeletal muscle mitochondria. However, in tissues with low L_4/L_3O ratio, such as smooth muscle and myoblasts, there was less cristae alignment (Fig. 7, D and E). Even auricular mitochondria (Fig. 7 B), although being derived from heart, had wider cristae sacks with some irregularities, such that they did not achieve the same kind of stacking seen in ventricle and skeletal muscle mitochondria.

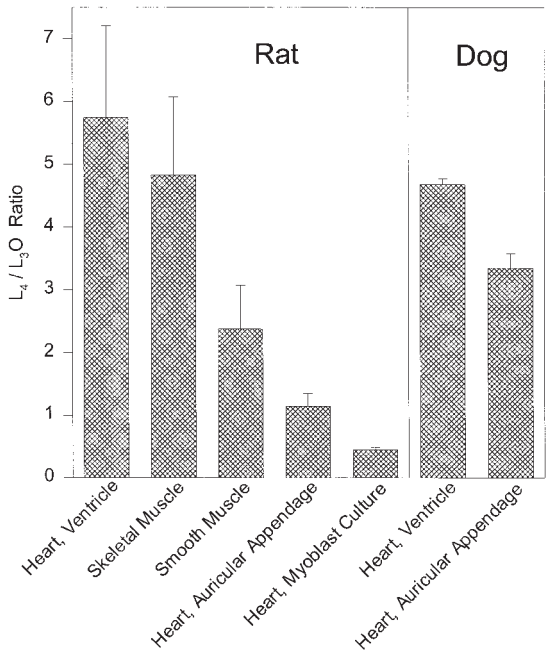


Fig. 6. L_4/L_3O ratio in various muscle tissues. Cardiolipins from various muscle tissues and from H9c2 myoblast cell culture were analyzed and the ratios of tetralinoleoyl-cardiolipin (L_4) to trilinoleoyl-oleoyl-cardiolipin (L_3O) were determined.

Skeletal muscle cardiolipin from patients with mitochondrial disease

Cardiolipin was measured in skeletal muscle biopsies from patients with mitochondrial disease and in control subjects (**Fig. 8**). There was no significant difference between control biopsies from five children and twelve adults. In the disease group, five out of eight patients (two patients with cytochrome oxidase deficiency, two patients with MELAS, and one patient with Leigh syndrome) had elevated cardiolipin content in skeletal muscle. The difference in cardiolipin content between seventeen control subjects and eight patients with mitochondrial disease was statistically significant ($P = 0.00326$).

The pattern of cardiolipin species in skeletal muscles from patients with mitochondrial disease is shown in **Table 1**. L_4 was the predominant species in all biopsies. The L_4/L_3O ratios were 4 to 6 in control biopsies and in biopsies from patients with cytochrome oxidase deficiency. However, in two patients with MELAS and in one patient with multiple deletions of the mitochondrial DNA, the proportion of L_4-cardiolipin was significantly lower in favor of more saturated species (L_4/L_3O ratios 2 to 4).

DISCUSSION

In this paper we describe a microanalytical method for the quantitation of cardiolipin. The procedure involves formation of a fluorescence derivative and its isolation by a series of extractions. Quantitation was based on fluorescence–HPLC in reference to an internal standard. The standard compound, tetrastearoyl-cardiolipin, is a synthetic analog that is uncommon in biological materials (2–4, 21). This assay is specific for cardiolipin and is, therefore, not suitable for the measurement of other phospholipids. As expected for fluorescence analysis, the assay was very sensitive. With the present array of HPLC instruments, one nanomole of cardiolipin produced a signal of approximately 100 mV, putting the lower limit of detection in the range of 10 to 20 pmol (corresponding to 1–2 mV on a total scale of 1.0 V). In practice, however, it was difficult to measure such low quantities for two main reasons: *i*) the recovery of cardiolipin was only 18–20%;

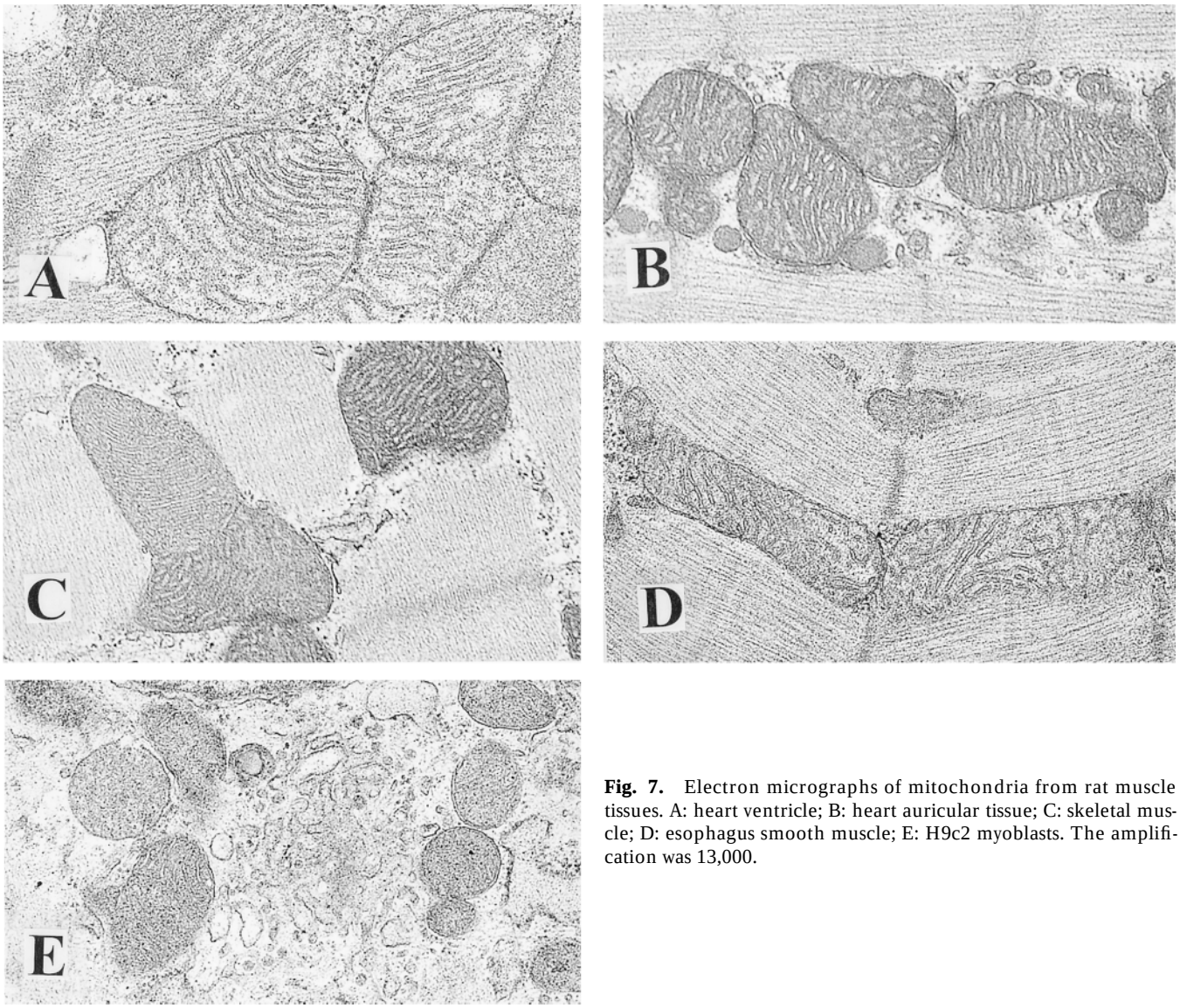


Fig. 7. Electron micrographs of mitochondria from rat muscle tissues. A: heart ventricle; B: heart auricular tissue; C: skeletal muscle; D: esophagus smooth muscle; E: H9c2 myoblasts. The amplification was 13,000.

and *ii*) only 10–20% of a total sample could be injected into the HPLC to avoid overloading with byproducts of the chemical derivatization. Thus, no reliable signal was obtained in biological samples with less than 0.5 nmol of cardiolipin. The cardiolipin concentration, measured by this method, correlated well with conventional phospholipid measurements made by colorimetric determination of phosphate after ashing (27).

The present method also allowed quantitation of individual molecular species of cardiolipin, which were resolved by HPLC. A similar separation of molecular species was achieved by a related technique, in which cardiolipin was converted to the 2-benzoyl-cardiolipin dimethyl ester (28). As in the previous method, there was a logarithmic dependence of the HPLC retention time on the number of double bonds in cardiolipins in which all acyl chains had 18 carbon atoms (Fig. 3). Presumably, a similar relationship can be found for cardiolipins with other chain lengths but appropriate molecular species were not available to test this. The relationship between retention time and number of double bonds helped to identify species of muscle cardiolipins in routine analyses because they consisted almost entirely of C_{18} chains. Compared to the previous method (28), the new technique has a higher sensitivity and a higher specificity, whereas the resolution of molecular species is similar in the two methods. The increase of sensitivity and specificity is due to the use of fluorescence detection as well as the much improved sample work-up relying on specific extraction of derivatized cardiolipin.

In different muscle tissues, the concentration of cardiolipin (Fig. 4) corresponded roughly to the activity of cytochrome oxidase (data not shown), confirming that cardiolipin is an indicator of mitochondrial content in the tissue. The predominant molecular species in all muscles examined were L_4-cardiolipin and L_3O-cardiolipin. This applied to heart muscle (from rat and dog), skeletal muscle (from humans and rat), and smooth muscle (from rat) alike. However, there were significant differences in the L_4/L_3O ratio in different types of muscle (Fig. 6). While in rat heart ventricle and in rat skeletal muscle the L_4/L_3O

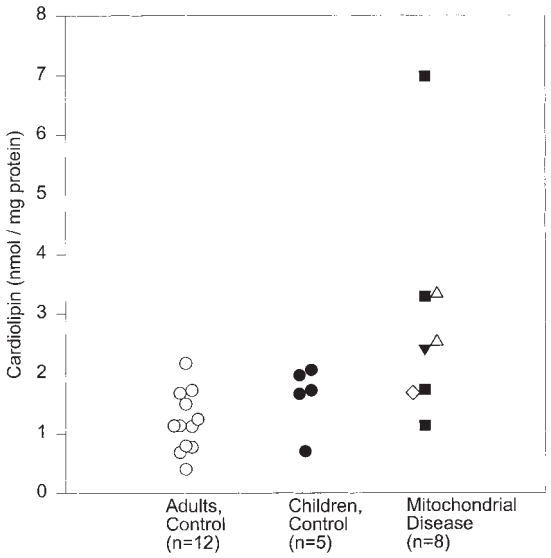


Fig. 8. Cardiolipin content in skeletal muscle from patients with mitochondrial disease and controls. Cardiolipin content was measured in skeletal muscle biopsies from adults (open symbols) and children (closed symbols). Patients with mitochondrial disease had cytochrome oxidase deficiency (squares), A3243G mutation in the $tRNA^{Leu(UUR)}$ gene resulting in MELAS (upward triangles), T8993G mutation of mitochondrial DNA resulting in Leigh syndrome (downward triangle), and multiple deletions of mitochondrial DNA (diamond).

ratio was about 5, in smooth muscle it was only 2, and in the auricular appendage of rat heart it was about 1. Most striking was the difference between ventricle and auricular appendage as both were derived from heart tissue. This difference was also found in dog, although to a lesser degree. In the H9c2 rat myoblast cell line, which is derived from embryonic heart tissue (29), the L_4/L_3O ratio was even lower than in the auricular appendage.

Mitochondria with a high L_4/L_3O ratio, such as those from heart ventricle and skeletal muscle, showed a condensed conformation with stacked cristae ("muscle" type mitochondria). In contrast, mitochondria from the auricular appendage of rat heart, H9c2 myoblasts, or smooth muscle, which had a low L_4/L_3O ratio, were characterized by an orthodox conformation with more relaxed cristae ("liver" type mitochondria). In rat liver the L_4/L_3O ratio was 2.2 ± 0.3 ($n = 3$). These data suggest that tissues with more condensed mitochondria, reflecting a higher oxidative metabolism, need a higher proportion of L_4-cardiolipin. This, in turn, is consistent with the idea that the function of L_4-cardiolipin is related to the density of packing in the cristae membrane. The cristae membrane has to accommodate the multimeric complexes of oxidative phosphorylation, plus, perhaps, an unknown number of regulatory and assembly proteins, resulting in such high protein concentration that it can be regarded as a lipoprotein (3).

TABLE 1. Composition of cardiolipin in skeletal muscle from patients with mitochondrial disease

Molecular Species	Composition			
	Control	COX Deficiency	MELAS 3243	Multiple Deletions
L_4	74.8 ± 3.9	77.5 ± 2.9	61.5 ± 3.4	60.2
L_3O	17.4 ± 2.1	13.3 ± 1.7	20.9 ± 3.5	22.5
L_2O_2	5.8 ± 1.1	4.7 ± 0.6	9.3 ± 2.4	10.4
Other	1.9 ± 1.2	4.5 ± 1.7	8.3 ± 2.6	6.9

COX, cytochrome oxidase.

Mitochondrial diseases are very hetereogeneous in clinical manifestation, but are often associated with typical morphological features in skeletal muscle (30, 31). In adults, the most prominent of these features is the presence of "ragged-red fibers", which are due to mitochondrial proliferation under the sarcolemma. Mitochondrial proliferation seems to be a futile attempt to compensate for single or multiple respiratory chain enzyme defects. We found that cardiolipin, the only specific mitochondrial phospholipid, is enriched in muscle biopsies from some patients with a mitochondrial disease. This finding is consistent with reactive proliferation of mitochondrial membranes. However, increased tissue concentration of cardiolipin was not observed in all patients. The variability within the disease group was much higher than in the control group, probably reflecting variations between specific defects or disease stages.

Interestingly, we found a low proportion of L_4-cardiolipin in three patients with mitochondrial DNA mutations. As the L_4/L_3O ratio is related to cristae structure in rat muscle tissues, a low L_4 proportion in these patients may be due to alterations of the inner mitochondrial membrane. One possible mechanism is that dysfunctional mitochondria were unable to synthesize enough L_4-cardiolipin to match the growth of cristae membranes. L_4-cardiolipin is probably the end-product of a fatty acid remodeling cycle (32). Alternatively, L_4-cardiolipin could have been degraded after oxidative damage, to which it is more vulnerable than other cardiolipin species. This latter hypothesis is consistent with accumulating evidence that defects in the respiratory chain are accompanied by increased levels of free radicals.

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