JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Partitioning of Exogenous δ-Tocopherol between the Triacylglycerol and Membrane Lipid Fractions of Chicken Muscle

HALLDOR SIGFUSSON*,† AND HERBERT O. HULTIN‡

Food and Agricultural Products Research & Technology Center, Oklahoma State University, Stillwater, Oklahoma 74078-6055, and Massachusetts Agricultural Experiment Station, Department of Food Science, University of Massachusetts/Amherst, Marine Station, Gloucester, Massachusetts 01930

The partitioning of exogenous δ-tocopherol, added dissolved in ethanol, between the neutral triacylglycerols and membranes of chicken leg muscles was investigated. The two lipid fractions were separated using differential ultracentrifugation techniques. Triacylglycerols were obtained after high-speed centrifugation of the minced muscle at 130000*g* for 30 min. Membranes were collected from a muscle−buffer homogenate (pH 7.5) between 10000*g* for 20 min and 130000*g* for 30 min. The triacylglycerols collected represented from 15 to 80% of the total triacylglycerols of the minced muscle, the yields increasing with increasing muscle triacylglycerol content. The phospholipids in the isolated membrane fraction represented from 20 to 35% of the total phospholipids of the muscle. At low muscle total lipid contents (3−5%), the added δ-tocopherol was present in approximately the same concentration in both muscle lipid fractions. At higher total lipid contents, achieved by adding exogenous triacylglycerols, the δ-tocopherol concentration in the membranes increased relative to that in the triacylglycerols.

KEYWORDS: Tocopherol; triacylglycerols; membrane lipids; partitioning; muscle

INTRODUCTION

Lipids of minced or comminuted muscle foods are particularly susceptible to oxidative deterioration (*1*). Moreover, the membrane phospholipids are more susceptible to oxidative changes than the neutral triacylglycerols (*2*). Antioxidants are added to muscle foods to retard the undesirable changes accompanying oxidation (*3*), most commonly along with flavors/spices, or in an inert solvent or carrier such as oil or propylene glycol (*4*). The levels of antioxidant addition are regulated by the U.S. Food and Drug Administration.

Antioxidants may function in the aqueous or lipid phases of the cell. Directing an added lipid-soluble antioxidant to the site where the lipids are most susceptible to oxidation is important. The exact location of the lipid-soluble antioxidant, its concentration, the ease with which it donates hydrogen to lipid radicals, and the stability of the resulting antioxidant radical determine the antioxidant efficiency. The choice of antioxidant will therefore depend on its chemical and physical characteristics, its effectiveness, its affinity for the lipid phase, its concentration in the various lipid phases, and its stability during and after processing (*5*). Vitamin E (tocopherol) is a natural lipid-soluble antioxidant commonly found in and added to muscle foods. In cellular membranes, tocopherol is located in both halves (leaflets) of the membrane bilayer, with its hydrophobic tail intercalated with the membrane fatty acids and the headgroup located near the lipid−water interface due to the polarity bestowed by its hydrophilic groups (*6*, *7*). This particular orientation is important, both for its membrane stabilizing effect (*8*) and for its antioxidant effect, as it scavenges radicals near the surface more efficiently than those formed in the inner region of the membrane (*9*).

The partitioning of antioxidants has been studied in various model or simple food systems (*10*−*12*). These include butteroil−water, lipid or surfactant micellar systems, or liposomal or membrane systems. Results have shown that the partitioning in these model systems is influenced by the properties of both the antioxidant and the lipid surface (*12*−*14*). However, no information is available in the literature on partitioning of exogenous antioxidants between the different lipids of complex systems such as muscle foods. The objective of this study was to determine the distribution of an exogenous lipid-soluble antioxidant, δ-tocopherol, when added to minced chicken leg muscles. The partitioning between the muscle triacylglycerol and membrane lipid fractions was studied in muscle ranging from approximately 3 to 15% (w/w) total lipids. Leg muscles were chosen because they have a higher membrane lipid (phospholipid) content than breast muscle. Exogenous triacylglycerols were added to lean muscle to increase the total lipids from their normal concentration (approximately 3−5% w/w) to levels that represent the levels found in commercial meat

* To whom correspondence should be addressed. Tel.: (405) 744-4590. Fax: (405) 744-6313. E-mail: sigfuss@okstate.edu.
† Oklahoma State University.
‡ University of Massachusetts/Amherst.

10.1021/jf0203491 CCC: $22.00
Published on Web 10/19/2002

products. It is a common practice in the industry to add adipose tissue for improved palatability/flavor. For each experiment, two to three levels of total lipids were compared. Only δ-tocopherol was added to samples since it is only a minor component of mammalian muscle lipids (*15*); this considerably simplifies data analysis and interpretation.

MATERIALS AND METHODS

Materials. Live hens of varying age were obtained from a local farm. HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer hemisodium salt), Nagarse type XXVII protease (EC 3.4.21.62), high-molecular-weight protein standard (30−200 kDa) for SDS−PAGE analysis, and δ-tocopherol (90% pure) were obtained from Sigma Chemical Co. (St. Louis, MO). High-purity α- (99%), γ- (99%), and δ-tocopherols (94%) for standard curve preparation were purchased from Supelco, Inc. (Bellefonte, PA). Whatman polytetrafluoroethyl acetate (PTFE) membrane filters (0.45 μm pore size) were obtained from Fisher Scientific (Pittsburgh, PA). Ethanol (dehydrated, 200 proof) was obtained from Pharmaco Products Inc. (Bayonne, NJ). Polyacrylamide gradient gels (4−20%) for SDS−PAGE analysis and staining kit (Owl Pro Blue) were obtained from Owl Separation Systems (Woburn, MA). All reagents were of ACS grade and all solvents of HPLC grade unless otherwise noted.

Muscle Sampling and Treatment. Chickens were sacrificed by asphyxiation using carbon dioxide. The legs were removed and deboned, and the thigh and drumstick muscles were collected. The muscles were stored whole at 0−4 °C until used (10−16 h). Postrigor muscles were used in all experiments. Prior to the addition of the antioxidant system, the stored muscles were ground twice through 5-mm perforations in a KitchenAid model KSM90 grinder (300 W) at full speed (KitchenAid Inc., St. Joseph, MI). Triacylglycerol contents of muscle samples were increased by adding isolated muscle triacylglycerols to ground muscle followed by mincing for 30 s in an Oskar Sunbeam model 4817 food processor (Sunbeam-Oster Household Products, Hattiesburg, MS). Muscle triacylglycerols were obtained by mincing and heating chicken depot fat for 30 min in a thermostated water bath at 45 °C. This was followed by centrifuging the oil obtained for 5 min at full speed at room temperature in an IEC clinical tabletop centrifuge (International Equipment Co., Needham Heights, MA) for better separation. The clear triacylglycerol layer was collected and added to lean ground muscle to increase total lipids. All samples were stored on ice throughout the experiments.

Isolation of Membrane Lipids. *Addition of Antioxidant.* The antioxidant system used consisted of sodium ascorbate (0.2% w/w) and δ-tocopherol (approximately 300 ppm on muscle total lipid basis). Sodium ascorbate and a proteolytic enzyme preparation (Nagarse) were added to ground muscle at levels of 0.2 and 0.005% (w/w), respectively. This was followed by mincing samples for 20 s in the Oskar Sunbeam food processor. The minced muscle was then incubated for 60 min at refrigerated conditions (0−4 °C). The enzyme was added to break down structural proteins to release membrane material (*16*−*18*). Ascorbate was added to provide protection to the added δ-tocopherol. After incubation, the appropriate volume of approximately 4.5 mM freshly made δ-tocopherol stock in ethanol (to give approximately 300 ppm on a lipid basis) was added to the minced muscle, followed by further mincing for 20 s in the Oskar Sunbeam food processor. The actual δ-tocopherol concentration of the stock was determined using HPLC. The stock was found to contain approximately 1 and 4% (w/w) of α- and γ-tocopherol, respectively. Immediately after addition of the tocopherol stock, muscle specimens were taken for chemical analysis.

Isolation of Membrane Lipids. The method of McDonald and Hultin (*19*) for the isolation of flounder microsomes was modified to isolate membranes from chicken muscle. Four volumes of a cold 0.1 M HEPES buffer (pH 7.5) containing 0.2% (w/v) ascorbate were added to minced muscle, followed by homogenization for 60 s in a Kinematica GmbH type PT 10/35 Polytron (Brinkmann Instruments, Westburg, NY) equipped with Adjust-A-Volt variable-speed control type 500B (Standard Electronic Products Co., Dayton, OH) set at speed 76. The pH of the homogenate was adjusted to 7.5 using 0.1 M NaOH, followed by centrifugation at 10000*g* for 20 min at 5−10 °C. The resulting supernatant was centrifuged at 130000*g* for 30 min at 5−10 °C, and the sediment thus obtained was suspended in 0.6 M KCl in the aforementioned HEPES buffer. This suspension was then centrifuged again at 130000*g* for 30 min at 5−10 °C, and the final sediment was gently suspended in the HEPES buffer. The high-ionic-strength buffer at neutral pH solubilized actomyosin, thus aiding in its removal. The chemical composition of the unwashed and salt-washed membrane fractions was analyzed as described below. For comparison, the chemical composition of chicken muscle microsomes isolated as described by McDonald & Hultin (*19*), but using the buffers mentioned above, was determined. Ascorbate (0.2% w/v) was added to all buffers used for protection of the added δ-tocopherol. All samples were kept on ice throughout the experiments.

Isolation of Triacylglycerols. Triacylglycerols were isolated according to Liang (*20*) as follows: duplicate 50 g minced muscle specimens were centrifuged in a Beckman L5-65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) at 130000*g* for 30 min at 35−40 °C. After centrifugation, the top oil layer was collected and pooled, transferred to a test tube, and recentrifuged at full speed for 5 min at room temperature in an IEC Clinical tabletop centrifuge (International Equipment Co.) for further separation. The oil collected was weighed, and the yields were estimated by comparison to the total amount of triacylglycerols in the muscle (estimated as the difference in the muscle total lipid and phospholipid contents). Liang (*20*) previously showed that there was no phospholipid contamination in the triacylglycerols obtained using this procedure. Triacylglycerol samples obtained were dissolved in chloroform, and the tocopherol content was determined using HPLC.

Chemical Analyses. The moisture content of minced muscle was determined by weight after drying under IR light in a CSC Scientific Moisture Balance (Fairfax, VA) at the highest setting for 20 min, or until no change in weight was observed. Protein contents of minced muscle and microsomal and membrane suspensions were determined by means of the Biuret reaction (*21*). Total lipids (TL) of minced muscle and membrane suspensions were determined by weight after extraction with a chloroform−methanol solvent according to the method of Lee et al. (*22*). A 1:1 (v/v) solvent combination was used to extract lipids of lean muscle and a 2:1 combination for fatty muscle. Phosphorus contents of lipid extracts and triacylglycerol samples were determined spectrophotometrically according to the method described by Anderson and Davis (*23*). A factor of 25 was used for converting lipid phosphorus to phospholipids, based on an average molecular weight of phosphatidylcholine divided by the atomic weight of phosphorus. Tocopherol contents of lipid extracts and triacylglycerol samples were determined as described by Petillo et al. (*24*) using an excitation wavelength of 295 nm and an emission wavelength of 325 nm. Lipid extracts were filtered through 0.45 μm pore size PTFE disk filters to remove particulate debris prior to injection to the HPLC. Triacylglycerol samples were dissolved in chloroform. Samples were stored in amber HPLC vials at −40 °C until injection into the HPLC. Values are reported as the average of measurements on at least duplicate samples.

SDS−PAGE Analysis. SDS−PAGE sample buffer was added to microsomal and membrane suspensions (2 mg mL^{-1}), followed by heating in a boiling water bath for 2 min and a quick cooling on ice. The samples were then stored at −40 °C until used. After thawing, samples were placed on 10 × 10 cm^2, 4−20% gradient polyacrylamide MiniPlus gels and run at a constant current of 30 mA per gel for approximately 60 min. This was followed by fixing gels in a 12% (w/v) trichloroacetic acid solution for 60 min, prestaining for 60 min, and staining overnight using the Owl Pro Blue kit (SE140002). After being rinsed with distilled deionized water, gels were scanned using a GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). The molecular weights of the membrane proteins were determined by comparison to molecular weight standards. The concentration of each protein was determined from the relative peak areas.

Statistical Analysis. Statistical analyses were carried out using the general linear model (GLM) procedure (Jandel Scientific, San Rafael, CA). Least significant difference (LSD) tests at $p < 0.05$ were used to

Table 1. Comparison of Chemical Characteristics of a Microsomal Fraction Obtained from Nontreated Muscle and Membrane Fractions Obtained from Enzyme-Treated Muscle with or without a Salt Wash (0.6 M KCl) To Remove Myofibrillar Proteins[a]

membrane fraction[b]	$PL_{membrane}/TL_{membrane}$	$PL_{membrane}/Protein_{membrane}$	$PL_{membrane}/PL_{muscle}$
microsomal[c]	0.911 ± 0.052 a	0.463 ± 0.047 a	0.140 ± 0.088 a
membrane without salt wash	0.809 ± 0.030 b	0.313 ± 0.024 b	0.343 ± 0.040 b
membrane with salt wash	0.848 ± 0.033 a	0.390 ± 0.032 a	0.247 ± 0.045 c

[a] Numbers reported are the averages and standard deviations from duplicate measurements and expressed as weight ratios (w/w). Numbers within each column having the same letters are not significantly different ($p > 0.05$). See text for definition of ratios. [b] The abbreviations PL and TL represent phospholipid and total lipid contents, respectively. The subscripts refer to the membrane pellet (sediment) obtained after centrifugation, or to the muscle from which the membranes were obtained. [c] Refers to a membrane preparation obtained by the method of McDonald and Hultin (*19*).

separate means when significant differences were found. All experiments were done at least twice.

RESULTS

Isolation of Triacylglycerols. The triacylglycerols isolated from the minced chicken leg muscle represented up to 80% of the total triacylglycerols of the muscle. For lean muscle consisting of approximately 3−5% (w/w) triacylglycerols, the yields were as low as 11% of the total triacylglycerols initially in the muscle. However, as the triacylglycerol content was increased by the addition of depot fat (triacylglycerols), the yields of isolated triacylglycerols were generally between 50 and 80% of the total triacylglycerols of the muscle. Phosphorus was not detected in the isolated triacylglycerol fractions (data not shown), suggesting an insignificant phospholipid contamination.

Isolation of Membrane Lipids. To examine the "purity" of the isolated membrane preparation obtained using the modified procedure, the chemical compositions of a microsomal fraction obtained from non-enzyme-treated muscle according to the method of McDonald and Hultin (*19*) and from membrane preparations obtained from enzyme-treated muscle using the modified procedure were compared. The contamination by neutral lipids and myofibrillar proteins of the preparations was estimated by calculating the ratio of phospholipid to total lipid content ($PL_{membrane}/TL_{membrane}$), and the phospholipid to protein content ($PL_{membrane}/Protein_{membrane}$). A high $PL_{membrane}/TL_{membrane}$ ratio indicates a low neutral lipid contamination. Likewise, a high $PL_{membrane}/Protein_{membrane}$ ratio indicates a low myofibrillar protein contamination. The fact that both neutral lipids (e.g., cholesterol, tocopherol, ubiquinol, and triacylglycerols) and membrane-associated proteins are an integral part of the membrane structure complicates the assessment of the level of contamination. The $PL_{membrane}/TL_{membrane}$ and $PL_{membrane}/Protein_{membrane}$ ratios of *microsomes* obtained using the method of McDonald and Hultin (*19*) and the *salt-washed* membrane fraction obtained using the modified procedure were not significantly ($p > 0.05$) different (**Table 1**), suggesting a similar chemical composition of the two membrane fractions. However, the triacylglycerol and protein contents of the *unwashed* membrane fraction were higher compared to those of the other fractions, as indicated by the lower $PL_{membrane}/TL_{membrane}$ and $PL_{membrane}/Protein_{membrane}$ ratios. The amounts of phospholipid in the membrane preparations that were obtained from 100 g of original muscle were 132 ± 6 mg for the microsomes, 275 ± 6 mg for the unwashed preparation, and 198 ± 6 mg for the salt-washed preparation.

The yield of microsomal phospholipid obtained using the method of McDonald and Hultin (*19*) was only 14% of the total muscle phospholipids ($PL_{membrane}/PL_{muscle}$), as compared to up to 34% obtained using the modified procedure without the salt wash (**Table 1**). Thus, the enzyme treatment resulted in significantly higher phospholipid yields. The additional salt-washing step removed some proteins (20% w/w) and triacylglycerols (5% w/w). This change is reflected by the increase in the $PL_{membrane}/TL_{membrane}$ and $PL_{membrane}/Protein_{membrane}$ ratios, respectively. However, this increase in "purity" was accompanied by a significant loss in polar membrane lipids, as estimated by the $PL_{membrane}/PL_{muscle}$ ratio, from approximately 34 to approximately 25% of the total phospholipids initially in the muscle. To further evaluate the "purity" of the membrane fractions obtained using the modified procedure, SDS−PAGE was performed, and the resulting protein patterns were examined to assess contamination by myofibrillar proteins (**Figure 1**). In theory, proteins can bind molecules such as δ-tocopherol through hydrophobic interactions. The major proteins in the membrane fractions were of molecular weight of approximately 105 kDa (**Table 2**), corresponding to that of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (33−36% of the total protein), and a band of approximately 57 kDa (19%). A band of molecular weight of approximately 205 kDa was observed in the unwashed membranes, suggesting the presence of a small amount of myosin heavy chain. Therefore, membranes prepared with the salt wash were used throughout the rest of the experiments.

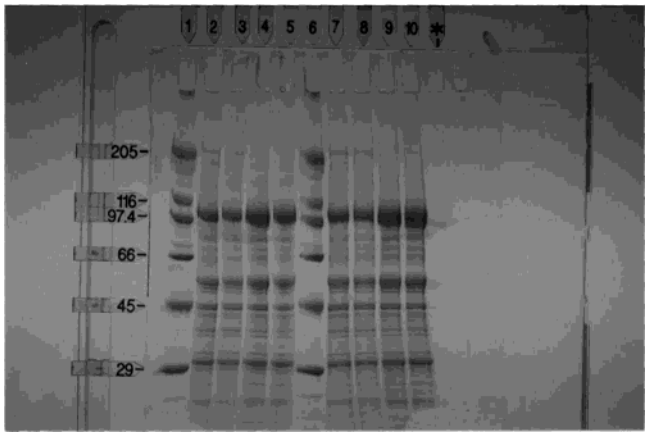

Figure 1. Protein patterns of membrane fraction isolated from enzyme-treated muscle with or without salt-washing to remove myofibrillar proteins. Lanes 1 and 6 are protein standards. Lanes 2, 3, 7, and 8 are unwashed membranes (18.80 μg loaded). Lanes 4, 5, 9, and 10 are salt-washed membranes (23.60 μg loaded).

Effect of Muscle Total Lipid Content on Partitioning of δ-Tocopherol. Chicken leg muscle of a wide range of total lipid contents was used in this study. Lean chicken leg muscle contained approximately 74 ± 3% moisture, 22 ± 2% protein, and 4 ± 1% total lipids (wet weight) of which approximately one-quarter were phospholipids (0.7−0.8% wet weight). The total lipid content was increased to approximately 14% by adding chicken depot fat (triacylglycerols) to the muscle. The partitioning of the added δ-tocopherol between the membranes

Table 2. Comparison of Protein Composition of Membrane Fractions Obtained from Enzyme-Treated Muscle with or without Washing To Remove Myofibrillar Proteins[a]

MW (kDa)	without salt wash (% of total protein)	with salt wash (% of total protein)
202–207	2	nd[b]
99–108	33	36
83–89	10	6
75–81	2	2
69–75	7	6
55–59	19	19
44–48	11	9
41–44	3	3
36–39	4	2
34–35	1	1
29–31	1	1
27–29	10	9

[a] A molecular weight (MW) range is given for each major band. Protein percentages are the average of quadruplicate determinations. [b] Not detected.

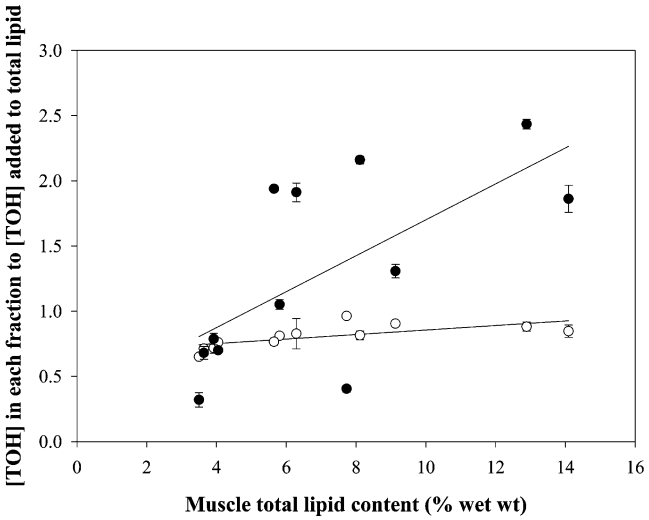

Figure 2. Partitioning of δ-tocopherol added in ethanol between triacylglycerols (TAG) and membrane lipids, depending on muscle total lipid content. Results are expressed as the tocopherol concentration measured in each lipid fraction compared to the concentration added to total lipids. (●) $[TOH_{membranes}]/[TOH_{added}]$; (○) $[TOH_{TAG}]/[TOH_{added}]$.

and triacylglycerols was estimated by comparison of the tocopherol concentrations determined in each lipid fraction after separation (**Figure 2**). As some δ-tocopherol (5−20% w/w) was destroyed upon addition to muscle and mincing, partitioning calculations were based on tocopherols that were recovered from minced muscle immediately after its addition. At a muscle total lipid content of approximately 3−5%, the distribution of the added δ-tocopherol between the membranes and triacylglycerols was approximately even on a concentration basis ($[TOH_{membrane}]/[TOH_{TAG}] \approx 1$). At higher total lipid contents, achieved by adding exogenous triacylglycerols, the δ-tocopherol uptake by the membranes increased relative to the triacylglycerols on a lipid weight basis. The δ-tocopherol concentration in the membranes was up to about 2 times that determined in the triacylglycerols at total lipid contents of approximately 12%. The uptake of tocopherol by membrane lipids depended on the amount of tocopherol added (**Figure 3**). Although the results were variable ($r^2 = 0.340$), there was a trend toward a higher tocopherol uptake by the membranes when the tocopherol added was increased.

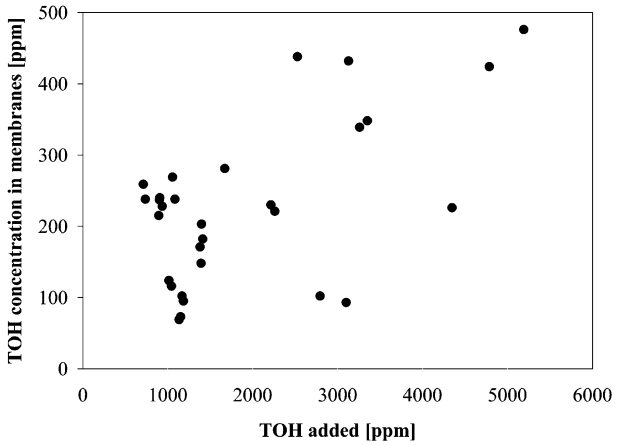

Figure 3. Tocopherol concentration measured in isolated muscle membrane lipids, depending on tocopherol added on a membrane lipid basis.

DISCUSSION

To estimate the partitioning of the exogenous tocopherol between the major muscle lipids with confidence, the lipid fractions obtained after separation should be not only of high yield but also of good purity, i.e., little contamination of membrane phospholipids in the triacylglycerols and vice versa. Using a centrifugation force of 130000*g* for 30 min at 35−40 °C, up to approximately 80% (w/w) of the total triacylglycerols of minced chicken leg muscle were obtained. The yields were based on gravimetric comparison of the amount of oil (triacylglycerols) obtained after centrifugation to the amount of triacylglycerols initially in the muscle. The yields increased with increasing total lipid content of the muscle. The triacylglycerol yields upon centrifugation of lean muscle (3−5% TL; ∼2−4% triacylglycerols w/w) ranged from 11 to 30% of the total triacylglycerols initially in the muscle. The yields increased to 80% for muscle of high lipid contents achieved by adding triacylglycerols. Liang (*20*) routinely obtained up to 50% (w/w) of the triacylglycerols of chicken leg muscle and up to 75% of the mackerel muscle triacylglycerols when applying 105000*g* centrifugation for 30 min at 30 °C. The triacylglycerol yields were shown to depend on the time of mincing the muscle and on the centrifugation temperature. Increasing the mincing time resulted in lower yields, probably due to the formation of a lipid−protein emulsion. Increasing the temperature resulted in higher triacylglycerol yields (*20*). It is possible that the exogenous triacylglycerols were relatively easier to isolate than the native triacylglycerols in the high-lipid muscle samples used in this study.

The membrane fraction obtained in these experiments most probably came from a variety of membrane sources, each of which has a different membrane lipid and protein composition. Phospholipids in a variety of membranes range from 60 to 90% of the total lipids of the membrane (*25*). The chemical properties (**Table 1**) of the salt-washed membranes obtained in this study using the modified isolation method were not significantly different ($p < 0.05$) from those of microsomes obtained using the method of McDonald and Hultin (*19*). However, the chemical characteristics of an unwashed membrane fraction indicated a slight contamination with triacylglycerols and myofibrillar proteins, as suggested by the lower PL/TL and PL/Protein ratios (**Table 1**). The PL/TL ratio of the salt-washed membrane fraction ranged from 0.8 to 0.95, slightly higher than the range of 0.70−0.75 for broiler microsomes reported by Asghar et al. (*26*). The PL/Protein ratio of the salt-washed

membrane fraction ranged from approximately 0.30 to 0.40. The TL/Protein ratio ranged from approximately 0.40 to 0.50, in agreement with the results of Yamauchi et al. (*27*) for chicken white muscle mitochondria (0.346) and for thigh muscle mitochondria (0.531). SDS−PAGE evaluation of the unwashed and salt-washed membranes revealed that the membranes obtained without the salt wash contained a small amount (1−4% w/w) of a polypeptide of a molecular weight of approximately 205 kDa, corresponding to that of myosin heavy chain. This band was not detected in the membranes obtained using the additional high-salt (0.6 M KCl) wash. Approximately 33−36% (w/w) of the total protein of the membrane fraction had a molecular weight corresponding to that of Ca^{2+}-ATPase (~105 kDa). This protein constitutes approximately 60−80% (w/w) of the total membrane proteins in the sarcoplasmic reticulum of skeletal muscle (*28*, *29*). Thus, it may be estimated that the SR protein constitutes some one-half of the total proteins isolated using the modified procedure.

The chemical characteristics of the membrane fractions obtained varied slightly with the muscle samples used for the various experiments. The characteristics of the membrane fractions isolated were independent of the muscle total lipid content. Muscle structural changes occurring postmortem may make a contribution to the variation in the chemical composition of the membrane fraction due to variability in muscle disintegration from one experiment to another. Muscle cell membranes are linked to myofibrillar proteins via several structural proteins such as utrophin, dystrophin, and desmin (*30*, *31*). Some of these proteins may still be linked to the cell membranes obtained after the ultracentrifugation procedure, thus affecting the overall chemical composition of the membrane fraction. The chicken muscle used was stored from 10 to 16 h at 0−4 °C prior to separating the muscle lipids.

Up to approximately 35% (w/w) of the total membrane lipids of the original minced chicken leg muscle were obtained using the modified differential centrifugation method of McDonald and Hultin (*19*). The yields were estimated on the basis of comparison of the absolute amount (milligrams) of lipid phosphorus obtained by physical separation of the membranes to the amount of lipid phosphorus in the original minced muscle after extraction of the lipid by organic solvents. The yields decreased to approximately 25% (w/w) upon washing the membrane sediment with 0.6 M KCl in 0.1 M HEPES buffer to remove contaminating myofibrillar proteins. For comparison, only approximately 10−17% (w/w) of the total membrane lipids were obtained using the original method of McDonald and Hultin (*19*). These yield estimates are based on the assumption that all of the lipid phosphorus extracted from muscle comes from cell membranes. However, Takahashi and colleagues (*30*, *32*) have reported that lipids are a significant component of Z-disks of vertebrate skeletal muscle (beef, pork, chicken, rabbit) and the lipid content varies depending on the muscle phenotype. Chicken (*Pectoralis profundus)* muscle Z-disk was found to contain approximately 2.4 g of lipids per 100 g of myofibrillar protein (lipids extracted from the I-Z-I brush only), of which 54% were phospholipids, 35% triacylglycerols, 7% cholesterol, and 5% free fatty acids. The chicken *semitendinosus* muscle Z-disk contained 3.9 g of lipids per 100 g of myofibrillar protein, of which 54% were phospholipids (*32*). It would seem likely that these phospholipids are not in membranes. Thus, it is possible that the lipid phosphorus extracted from the original tissue does not solely represent membrane phospholipids, and the percentage yield of membrane may therefore actually be higher. For example, if we assume that 100 g of original tissue has 20 g of myofibrillar protein and that there is approximately 3.9 g of lipids in the Z-disk of 100 g of myofibrillar protein (chicken *semitendinosus*), of which approximately 54% is phospholipid, this corresponds to approximately 0.42 g of phospholipid. This represents one-half of the total phospholipids found in the original tissue (0.7−0.8% wet weight) and is therefore quite significant in terms of recovery of membranes from muscle. As an example, a 35% yield would convert to a 70% yield under these conditions.

Comparison of the tocopherol concentrations (ppm) determined in each lipid fraction after separation suggested an approximately even distribution of tocopherol between the lipids when the muscle total lipid content was low (3−5%). However, as the muscle total lipid content was increased by adding triacylglycerols (and thus the amount of tocopherol added since it is added on a total lipid weight basis), the tocopherol concentration in the membranes increased relative to that in the triacylglycerols (**Figure 2**). This is based on regression analysis of the data in **Figure 2**, yielding a slope of 0.138 ($r^2 = 0.436$) for the membrane, and a slope of 0.017 ($r^2 = 0.473$) for the triacylglycerols. This result can be explained by kinetic considerations. The δ-tocopherol is added in a relatively polar solvent, ethanol, with a dielectric constant of 25.3 (*33*). The hydrophilic nature of this carrier, in combination with the polar headgroup of the tocopherol molecule, would be compatible with the hydrophilic surface of the membranes and may transfer the tocopherol more rapidly into the membrane lipids than across the more hydrophobic surface of the triacylglycerol oil droplets. When the δ-tocopherol:ethanol solution enters the aqueous phase of the muscle tissue, the ethanol is immediately diluted. Due to the small amount of ethanol added to the muscle (<1% w/w), the δ-tocopherol would rapidly become insoluble and most likely form micelles or tiny droplets of tocopherol. These would have the polar headgroups directed toward the aqueous phase. This might make them react more rapidly with the polar surface of the membrane than with the more hydrophobic oil droplets. With time, δ-tocopherol concentration in the membrane would increase, which would slow the uptake of additional antioxidant molecules into the membrane. If the initial rate of tocopherol uptake were dependent on the aqueous concentration of tocopherol added, i.e., first order, an increased uptake of δ-tocopherol by the membranes compared to the oil phase would occur if the tocopherol content of the aqueous phase of the meat increased. This is the case as tocopherol is added, based on the total lipid content (300 μg/g of lipid): as the total lipid content of the muscle increases, increasing *amounts* of tocopherol are added, resulting in a high aqueous concentration. The net effect would be a higher concentration of δ-tocopherol in the membranes as the total lipid (and therefore also δ-tocopherol) content of the system increased.

For comparison, the partitioning data presented in **Figure 2** were also expressed in terms of absolute amounts (micrograms) of tocopherol measured in each lipid fraction as opposed to tocopherol concentrations. The absolute tocopherol amounts in each lipid fraction were calculated on the basis of the assumption that the lipids isolated are representative of all of each type of lipid in the muscle, and thus the tocopherol concentration determined in the isolated lipid fractions is the same as that of the lipids that still remain in the muscle. To demonstrate the trend observed for the antioxidant partitioning with increasing muscle total lipid content, examples of the results from **Figure 2** are shown in **Table 3** on an absolute weight basis. For ease of presentation, results for 3 of a total of 30 samples are shown.

Table 3. Example of Results for Absolute Distribution of Added δ-Tocopherol between Triacylglycerols (TAG) and Membrane Lipids in Minced Muscle of Varying Total Lipid Contents[a]

	muscle total lipid content (% wet weight)		
	3.6%	5.8%	12.9%
total TOH in 100 g of muscle[b]	900 ± 14	1473 ± 6	2645 ± 67
in triacylglycerols (TAG)	493 ± 6 (54.8%)	1027 ± 5 (69.7%)	2218 ± 25 (83.9%)
in membranes	142 ± 7 (15.8%)	214 ± 6 (14.5%)	311 ± 6 (11.8%)
sum in TAG + membranes	635 (70.6%)	1241 (84.2%)	2529 (95.6%)

[a] Values reported are calculated on the basis of average ($N = 2$, $n = 2$) lipid contents of 100 g of minced muscle. Numbers in parentheses represent the fraction of the total tocopherol recovered. [b] Amount of tocopherol recovered from muscle immediately after its addition.

The membrane yields for the three samples presented were approximately 27, 30, and 25%, and the triacylglycerol yields were approximately 34, 53, and 71%. On an absolute weight basis, the majority of the exogenous tocopherol recovered from the muscle immediately after its addition was found to be associated with the triacylglycerols, or approximately 55% (w/w) for the lean sample (3.6% total lipids). For the medium-lipid sample (5.8% total lipids), approximately 70% of the tocopherol recovered was associated with the triacylglycerols, and approximately 84% for the high-lipid (12.9% total lipids) sample. The triacylglycerols are the bulk of the total lipids of the muscle, or 77, 86, and 95% of the total lipids of the three samples, respectively. The partitioning therefore seems to reflect what would be expected in a competitive situation where there is a majority of one component. The antioxidant becomes primarily associated with the major component. It is worth noting that it will take a large amount (micrograms) of tocopherol to result in a significant change in its concentration (micrograms per gram of lipid) in the major component, whereas a small change in the amount of tocopherol in the minor component (the membrane lipids) results in a very large percentage increase in its concentration.

Furthermore, higher amounts of tocopherol were incorporated into the two lipid fractions as the total lipid content increased (**Table 3**). The amounts increased from approximately 71% for the lean sample to approximately 96% for the high-lipid sample. Thus, in lean muscle, although the added tocopherol could be recovered by solvent extraction, a significant proportion could not be found in the isolated lipid fractions. This could mean that the tocopherol was in the aqueous phase or that some of the membrane or neutral lipids that remained in the muscle contained a greater amount of the antioxidant than the lipids that were isolated. The lower recovery of tocopherol from muscle of low lipid content immediately after its addition may indicate that the added tocopherol goes more easily into the triacylglycerol fraction.

There is some uncertainty about the uniformity of antioxidant distribution within each of the fractions due to the incomplete recovery of lipids in each of the two fractions examined. The relatively constant tocopherol uptake (based on concentrations) over a wide range of triacylglycerol concentrations would suggest that the isolated oil probably represents the total oil fractions. The situation with the membrane fraction is more problematic. However, there was a significant fraction of the membrane that took up added tocopherol at a concentration considerably greater than that which was added (based on lipid content). Furthermore, the antioxidant does not seem to pass readily between the two lipid fractions once it is taken up, as seen in studies in model systems (*34*) as well as in actual muscle systems (*35*). The lipids that initially contact the antioxidant appear to be critical in the final distribution of the antioxidant.

ACKNOWLEDGMENT

The authors thank Ms. Betsy Carter for valuable help in the preparation of the manuscript.

LITERATURE CITED

(1) Dawson, L. E.; Gartner, R. Lipid oxidation in mechanically deboned poultry. *Food Technol.* **1983**, *37*, 112−116.
(2) Pikul, J.; Leszczynski, D. E.; Kummerow, F. A. Relative role of phospholipids, triacylglycerols, and cholesterol esters on malonaldehyde formation in fat extracted from chicken meat. *J. Food Sci.* **1984**, *49*, 704−708.
(3) Chan, H. W.-S.; Coxon, D. T. Lipid hydroperoxides. In *Autoxidation of Unsaturated Lipids*; Coxon, D. T., Ed.; Academic Press: London, UK, 1987; pp 17−49.
(4) Chipault, J. R. Antioxidants for use in foods. In *Autoxidation and Antioxidants*; Lundberg, W. O., Ed.; Interscience Publishers: New York, 1962; pp 478−542.
(5) Buck, D. F. Antioxidant applications. *The Mfg. Confectioner* **1985**, p 45.
(6) Gomez-Fernandez, J. C.; Villalain, J.; Aranda, F. J.; Ortiz, A.; Micol, V.; Coutinho, A.; Berberan-Santos, M. N.; Prieto, M. J. E. Localization of α-tocopherol in membranes. *Ann. N.Y. Acad. Sci.* **1989**, *570*, 109−120.
(7) Bisby, R. H.; Ahmed, S. Transverse distribution of α-tocopherol in bilayer membranes studies by fluorescence quenching. *Free Radical Biol. Med.* **1989**, *6*, 231−239.
(8) Salgado, J.; Villaín, J.; Gómes-Fernándes, J. C. α-Tocopherol interacts with natural micelle-forming single-chain phospholipids stabilizing the bilayer phase. *Arch. Biochem. Biophys.* **1993**, *306*, 368−376.
(9) Takahashi, M.; Tsuchiya, J.; Niki, E. Mode of action of vitamin E as antioxidant in the membranes as studied by spin labeling. *Ann. N.Y. Acad. Sci.* **1989**, *570*, 521−523.
(10) Cornell, D. G.; DeVilbiss, E. D.; Pallansch, M. J. Partition coefficients of some antioxidants in butteroil−water model system. *J. Dairy Sci.* **1970**, *53*, 529−532.
(11) Castle, L.; Perkins, M. J. Inhibition kinetics of chain-breaking phenolic antioxidants in SDS micelles. Evidence that intermicellar diffusion rates may be rate-limiting for hydrophobic inhibitors such as α-tocopherol. *J. Am. Chem. Soc.* **1986**, *108*, 6381−6382.
(12) Schwarz, K.; Frankel, E. N.; German, J. B. Partition behavior of antioxidative phenolic compounds in heterophasic systems. *Fett/Lipid* **1996**, *98* (3), 115−121.
(13) Barclay, L. R. C.; Vinqvist, M. R. Membrane peroxidation: Inhibiting effects of water-soluble antioxidants on phospholipids of different charge types. *Free Radical Biol. Med.* **1994**, *16*, 779−788.
(14) Mei, L. M.; McClements, D. J.; Wu, J.; Decker, E. A. Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH and NaCl. *Food Chem.* **1998**, *61*, 307−312.

(15) Bauernfeind, B. C. The tocopherol content of food and influencing factors. *CRC Crit. Rev. Food Sci. Nutr.* **1977**, *8*, 337−382.
(16) Fleischer, S.; Klouwen, H.; Brierley, G. Studies of the electron-transfer system. XXVIII. Lipid composition of purified enzyme preparations derived from beef heart mitochondria. *J. Biol. Chem.* **1961**, *236*, 2936−2941.
(17) Luo, S.-W. J. NAD(P)H-dependent lipid peroxidation in trout muscle mitochondria. Ph.D. dissertation, University of Massachusetts, 1988.
(18) Rasmussen, U. F.; Rasmussen, H. N.; Andersen, A. J.; Jørgensen, P. F.; Quistorff, B. Characterization of mitochondria from pig muscle: Higher activity of exo-NADH oxidase in animals suffering from malignant hyperthermia. *Biochem. J.* **1996**, *315*, 659−663.
(19) McDonald, R. E.; Hultin, H. O. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *J. Food Sci.* **1987**, *52*, 15−21; 27.
(20) Liang, Y. Incorporation of antioxidants into the neutral lipid phase of minced muscle tissue. M.S. thesis, University of Massachusetts, 1998.
(21) Gornall, A. G.; Bardamill, C. J.; David, M. M. Determination of serum proteins by means of the Biuret reaction. *J. Biol. Chem.* **1949**, *177*, 751−766.
(22) Lee, C. M.; Trevino, B.; Chaiyawat, M. A simple and rapid solvent extraction method for determining total lipids in fish tissue. *J. AOAC Int.* **1996**, *79*, 487−492.
(23) Anderson, R. L.; Davis, S. An organic phosphorus assay which avoids the use of hazardous perchloric acid. *Clin. Chim. Acta* **1982**, *121*, 111−116.
(24) Petillo, D.; Hultin, H. O.; Krzynowek, J.; Autio, W. R. Kinetics of antioxidant loss in mackerel light and dark muscle. *J. Agric. Food Chem.* **1998**, *46*, 4128−4137.
(25) Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Molecular Biology of the Cell*, 2nd ed.; Garland Publishing: New York, 1989; p 281.
(26) Asghar, A.; Lin, C. F.; Gray, J. I.; Buckley, D. J.; Booren, A. M.; Crackel, R. L.; Flegal, C. J. Influence of oxidized dietary oil and antioxidant supplementation on membrane-bound lipid stability in broiler meat. *Br. Poultry Sci.* **1989**, *30*, 815−823.
(27) Yamauchi, K.; Nagai, Y.; Yada, K.; Ohashi, T.; Pearson, A. M. A relationship between α-tocopherol and polyunsaturated fatty acids in chicken and porcine skeletal muscle mitochondria. *Agric. Biol. Chem.* **1984**, *48*, 2827−2830.
(28) Madden, T. D.; Chapman, D.; Quinn, P. J. Cholesterol modulates activity of calcium-dependent ATPase of the sarcoplasmic reticulum. *Nature* **1979**, *279*, 538−541.
(29) Blazyk, J.; Wu, C.-J.; Wu, S.-C. Correlation between lipid fluidity and tryptic susceptibility of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes. *J. Biol. Chem.* **1985**, *260*, 4845−4849.
(30) Takahashi, K. Structural weakening of skeletal muscle tissue during post-mortem ageing of meat: The non-enzymatic mechanism of meat tenderization. *Meat Sci.* **1996**, *43*, S67−S80.
(31) Winder, S. J. The membrane−cytoskeleton interface: The role of dystrophin and utrophin. *J. Muscle Res. Cell Motil.* **1997**, *18*, 617−629.
(32) Takahashi, K.; Shimada, K.-I.; Ahn, D.-H.; Ji, J.-R. Identification of lipids as the main component of skeletal muscle Z-discs. *J. Muscle Res. Cell Motil.* **2001**, *22*, 353−360.
(33) *CRC Handbook of Chemistry and Physics*, 82nd ed.; Lide, D. R., Ed.; Boca Raton, FL, 2001; pp 6−154.
(34) Sigfusson, H.; Hultin, H. O. Partitioning of δ-tocopherol in aqueous mixtures of triacylglycerols and isolated muscle membranes. *J. Am. Oil Chem. Soc.* **2002**, *79*, 691−697.
(35) Davila, M. A. Distribution of delta-tocopherol between polar and neutral lipids of chicken leg muscle when added in solvents of different dielectric constants. M.S. Thesis, University of Massachusetts, 2001.

Received for review March 20, 2002. Revised manuscript received August 29, 2002. Accepted September 12, 2002. This material is based upon work supported by the Cooperative State Research, Extension, Education Service, U.S. Department of Agriculture, Massachusetts Agricultural Station, under Project No. MAS00759, and by Grant No. 94-37500-0513 of the NRI Competitive Grants Program/USDA. Approved for publication by the Director, Oklahoma Agricultural Experiment Station.

JF0203491