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Polyunsaturated fatty acids in mitochondria

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SUMMARY

Fatty acids of mitochrondria from hearts and livers of fresh-water and marine fish, marine birds, fur seal, and from sweet potato have been analyzed by gas-liquid chromatography. Cytochrome patterns, determined from difference spectra, were similar for all animal species; the quantities of cytochromes varied slightly, heart mitochondria yielding higher values than the liver mitochondria in each species. Fish mitochondria showed a slightly lower cytochrome content than bird and mammalian mitochondria, while sweet potato particles contained very little cytochrome. Fatty acid patterns and degree of unsaturation were similar in mitochondria from fish and from fish-eating birds and seals. Major differences found in fatty acid patterns included: absence of detectable linoleic acid in liver mitochondria from sturgeon and flounder; absence of detectable arachidonic acid in mitochondria from sweet potato; a four-fold greater percentage of stearic acid and a four-fold greater percentage of combined linoleic-arachidonic acids in bird and seal mitochondria compared with those found in fish mitochondria; high levels of the *linolenate* family of acids in fish mitochondria and intermediate levels in marine bird and seal mitochondria as compared to the high levels of the *linoleate* family of acids that have been found in rat liver, chicken liver, and beef heart mitochondria.

The biochemical function of the classical essential fatty acids (EFA), linoleic and arachidonic acids, is obscure. Generally, deficiency in rats results in dermal syndromes and growth inhibition. Both symptoms are cured by linoleic and arachidonic acids, whereas only growth is supported by linolenic acid and the fatty acids characteristic of fish oils (1–3). Specific structural requirements for support of growth are presumably lower since those fish fatty acids tested were apparently of the linolenic acid family and contained only a small percentage of fatty acids of the linoleic acid series (2, 3). The numerous demonstrations of metabolic insufficiency in mitochondria of EFA-deficient animals indicate that EFA are necessary for proper functioning of the mitochondria. EFA may be required for the proper structure of mitochondrial membranes (4) and/or for enzymic oxidation-reduction reactions involving the active methylene hydrogens (5, 6). Currently, the available experimental evidence supports the former hypothesis.

Fatty acid analyses of mitochondria from various sources might lead to a more generalized understanding of the fatty acids essential for the structure and function of mitochondria. This paper reports the fatty acid distribution in heart and liver mitochondria from fish and from marine birds and seals that exist on a natural diet of marine life. If the fatty acid composition of mitochondria faithfully reflects that of the diet, mitochondria from marine birds and seals that feed essentially on fish might have relatively low levels of EFA, reminiscent of "deficient" mitochondria (4), and high levels of polyunsaturated fatty acids. On the other hand, the homoiothermic organism may have different requirements and may modify the influence of the diet by incorporating fatty acids synthesized *de novo* or by concentrating the essential ones. This paper confirms a

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previous observation (7) that fish mitochondria do not necessarily contain linoleic and linolenic acids, and it reports the absence of detectable arachidonic acid in mitochondria from sweet potatoes.

METHODS AND MATERIALS

Organs, mainly hearts and livers, from marine birds numbering 10 California brown pelicans, *Pelicanus occidentalis*, 11 Brandt's cormorants, *Phallacrocorax penicillatus*, 6 California murres, *Uria aalge californica*, 7 surf scoters, *Melanitta perspicillata*, in addition to 5 fur seals, *Callorhinus ursinus*, were collected off the Northern California coast between the Farallon Islands and Drake's Bay. Organs from 6 western grebes, *Aechmophorus occidentalis*, were collected at Clear Lake, California. Organs from 30 petrale sole, *Eopsetta jordani*, 30 chili pepper, *Sebastodes goodei*, and 2 different species of rock fish, Genus *Sebastodes*, 7 designated as rock cod and 15 as cod, were caught off the Farallon Islands and obtained fresh from a commercial source in San Francisco within 24 hr of capture. Organs from 12 striped bass, *Roccus saxatalis*, 9 sturgeon, *Acipensis transmontanus*, and 65 starry flounder, *Platichthys stellatus*, were collected in Suisun Bay, California. In all cases, the organs were removed as soon as possible and maintained in cracked ice until mitochondria could be prepared. Generally, no more than 24 hr elapsed between collection of the organs and isolation of the mitochondria.

Mitochondria were isolated from representative samples of the appropriate tissue as described before (7). In addition, particles were isolated according to the method of Hackett et al. (8) from representative samples of 8 sweet potatoes. Fish livers that were especially fatty fractionated differently, yielding very little initial debris, and the supernatant layer over the mitochondrial pellet was highly opaque and fatty. A mitochondrial pellet was obtained, however, at a centrifugal force of 10,000 $\times$ *g* for 10 min.

The mitochondria were characterized by their sedimentation in sucrose solution and by quantitative estimation of their cytochrome content. Cytochromes were determined from difference spectra obtained with the Beckman DK-2 spectrophotometer after reduction with dithionite as previously described (9). The extinction coefficients and wavelength pairs of Chance and Williams (10) were used for quantification. Protein was determined by the biuret method of Gornall et al. (11). Extraneous free fat was removed from all mitochondria by washing with sucrose solution, usually 2 to 3 times. The packed mitochondrial pellets, generally isolated from 80 to 160 g of tissue, were saponified and treated as described by Holman and Widmer (12) to obtain the free fatty acids, which were then esterified in dry HCl–methanol as outlined by James (13) with a yield of 97 $\pm$ 3% esters. Samples of the esters were hydrogenated by the method of Farquhar et al. (14). The esters were analyzed by gas-liquid chromatography using 2 different instruments and detectors under different conditions. Esters obtained from commercial fish and sweet potato were analyzed on a Research Specialties Co. Model 600 instrument equipped with a beta-ionization detector. Columns were 6 and 10 ft, $^1/_4$-inch o.d. packed with 2.7% diethyleneglycol succinate on 80–100 mesh siliconized (15) Chromosorb W. The flash heater was set at 290° or 120° above the column temperature while the beta-ionization detector was maintained at 210°. Argon flow rate was 35 ml/min, and the sample volume was 0.05–0.25 μl. Because of instrumentation difficulties, the remaining ester samples were analyzed on an Aerograph Model A-90-C chromatograph equipped with a 4-filament thermal conductivity cell and a 6-ft, $^1/_4$-inch o.d. column packed with 15% diethylene glycol succinate on 60–80 mesh firebrick. Column temperature was 200 $\pm$ 2°, and the column preheater and outlet tube were respectively 60° and 50° above this temperature. Filament current was 230 ma, and chromatograms were recorded with a 1-mv full-scale recorder. Helium flow rate was 60 ml/min. Nearly all the solvent was evaporated from the normal and hydrogenated methyl ester sample before 1–2 μl of each was injected. Separations of saturated and unsaturated esters up to C22:6 were complete in 90 min.

Peaks were identified by comparing retention volumes, relative to methyl stearate, of known compounds with eluted unknowns, by comparing hydrogenated and nonhydrogenated samples, by preparing a logarithmic plot of relative retention time against chain length or degree of unsaturation for the various components, and by comparison of relative retention volumes with published data obtained under comparable conditions (16, 17). The area per cent for each component was determined by triangulation.

Correction factors for quantitative estimation of the mole percentages of the esters were obtained as explained previously (16). Briefly, an area correction factor for each ester was calculated by dividing its known weight percentage in an appropriate synthetic mixture of purified esters by the observed area. In the case of minor constituents for which factors were not available, the correction factors were estimated by interpolation between known values of neighboring esters. The area for each mitochondrial fatty acid ester was multiplied by its correction factor, and the

TABLE I. CYTOCHROMES IN MITOCHONDRIA

Mitochondria	Moles/mg Protein × 10^{10}			
	a	b	c	a_3
Fish Liver				
White Catfish	1.9	1.1	1.7	2.9
Bluegill	1.2	0.9	1.2	1.6
Chili Pepper	0.9	0.9	1.2	0.9
Rock Cod	1.1	0.2	0.3	1.4
Cod	0.3	1.1	1.2	1.2
Starry Flounder	0.8	0.3	0.5	1.1
Petrale Sole	0.3	0.1	0.3	...
Stripped Bass	0.2	...	0.3	0.3
Fish Heart				
Cod	3.3	1.5	2.1	6.1
Chili Pepper	2.6	0.9	1.8	4.0
Petrale Sole	2.3	0.5	1.2	3.5
Rock Cod	1.7	1.4	2.2	2.0
Bird Liver				
Cormorant	1.0	1.0	1.1	1.4
Surf Scoter	...	1.0	1.3	1.0
Brown Pelican	0.9	0.9	1.1	1.3
Calif. Murre	0.9	0.9	1.6	1.6
Western Grebe	0.6	0.8	1.2	1.3
Bird Heart				
Cormorant	5.3	3.1	4.4	7.1
Brown Pelican	5.1	3.3	2.9	6.5
Western Grebe	3.6	1.9	2.9	5.5
Bird Kidney				
Brown Pelican	0.8	0.3	1.4	1.9
Mammals				
Seal Heart	3.9	2.0	3.0	4.6
Seal Liver	0.8	1.0	0.8	1.2
Rat Liver	0.9	0.9	1.7	1.8
	Moles/g tissue × 10^{11}			
Sweet Potato	0.3	0.3	0.8	0.2

corrected areas were normalized by expressing each as a percentage of the total peak area. These data, representing weight per cent of each ester, were then converted to mole per cent of fatty acid.

RESULTS

Cytochrome analyses of mitochondria from all the species studied are listed in Table 1. In general, the cytochrome contents agree rather well with those recently published by Estabrook and Holowinsky (18) for mammalian mitochondria. Mitochondria from sturgeon liver contained a melanoid pigment which prevented estimation of the cytochromes. Values not listed were uncommonly low, apparently due to scattering effects and to slight spectral shifts that prevented good wavelength pairing.

This is the first characterization of mitochondria and cytochromes in these fish, marine birds, and the seal. Little is known of the intermediary metabolism of marine animals (19), and knowledge of cytochrome patterns of mitochondria is mainly limited to common mammals. Therefore, it is interesting to have these comparative data and note how similar the cytochrome patterns of these mitochondria are to those most extensively studied—namely, rat liver and beef heart mitochondria.

Yields of mitochondrial fatty acids, in general, averaged about 22 mg fatty acids per 100 mg protein. Fish liver mitochondria contain a slightly higher fatty acid content as compared to marine bird and mammalian mitochrondria with yields averaging 26 ± 3, 21 ± 2, and 18 ± 4 mg, respectively.

Tables 2 and 3 show the fatty acid analyses of fish, bird, sweet potato, and mammalian mitochondria. These data confirm a previous observation (7) that fish mitochondria contain very low levels of classical EFA, linoleic and arachidonic acids. In sturgeon liver and starry flounder liver mitochondria, there was no detectable linoleic acid. From the analytical curves, it is estimated that sturgeon liver mitochondria could not contain more than 0.1 mole % and flounder liver mitochondria not more than 0.2 mole % of linoleic acid. It is interesting to note that sweet potato mitochondria contain no detectable arachidonic acid.

As shown in Table 3, the mitochondria from birds and seals contain high levels of eicosapentaenoic and docosahexaenoic acids. Table 4, however, indicates that these same mitochondria contain levels of linoleic and arachidonic acids approximating those of the higher polyunsaturated fatty acids. By comparison, the relatively high eicosapentaenoic and docosahexaenoic acids and much lower EFA in fish mitochondria parallels the pattern for marine oils in general (20). This is reflected in the average total of 4% EFA for fish mitochondria, which is less than one-fourth that for the mitochondria of birds and seals. Another interesting comparative feature is the four-fold greater amount of stearate in the mitochondria of birds and seals as compared to those of fish.

The average degree of unsaturation in animal mitochondria, based on double-bond index (sum of the products of mole fraction of each acid and its number of double bonds) varied slightly from 1.96 for the bird to 2.21 for the seal mitochondria. The unsaturation of the fatty acids in the mitochondria of animals that eat fish approaches that of the fish at 2.01, as distinguished from rats, beef, and chickens, on rations containing vegetable oils and animal fats, with an average mitochondrial fatty acid unsaturation of 1.61, 1.27, and 1.15, respectively (7). Fatty acids from sweet potato mitochondria are much more saturated with a double-bond index of 0.83.

There was no striking difference in the distribution of the classes of fatty acids among the animal mitochondria. As shown in Table 5, bird and mammalian mitochondria had higher levels of saturated and lower levels of monounsaturated acid than fish mitochondria. Mammalian mitochondria showed percentages of polyunsaturated fatty acids somewhat higher than found in fish mitochondria.

The polyunsaturated fatty acids of rat, beef, and chicken mitocondria as compared to those of marine birds, seals, and fish vary greatly in the position of the double bonds. The higher polyunsaturated fatty acids of fish, and presumably those in fish-eating animals, are of the linolenate family (20), whereas the major polyunsaturated fatty acids in rat, beef, and chicken mitochondria are of the linoleate family. These differences are brought out in Table 6, which shows the mole ratio of the linoleate family to linolenate family in various mitochondrial fatty acid preparations. In fish, the linolenate series predominates, exceeding the linoleate series by a factor of about 10; in the rat, beef, and chicken, the linoleate series is favored by either the same or much greater margin. Marine birds and seals that feed on fish display an intermediate ratio.

DISCUSSION

In a nutritional sense, the distribution of fatty acids in the mitochondria of animals consuming fish and other marine life is interesting. We selected fur seals, cormorants, pelicans, surf scoters, and murres for this study because they are known to exist entirely on fish and other marine foods (21–23). This may be compared to the classical nutritional approach of feeding a domesticated animal a high fish diet. Advantages of selecting these wild animals of known dietary habits

TABLE 2. FATTY ACIDS IN MITOCHONDRIA (AREA %)

Fatty Acid*	Average Retention Volume	Rock Cod Liver	Rock Cod Heart	Sole Liver	Sole Heart	Cod Liver	Cod Heart	Chili Pepper Liver	Chili Pepper Heart	Sweet Potato
Unknown		0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	2.9
Unknown		0.0	0.0	tr	0.3	0.0	0.1	0.0	0.0	0.0
13:0	0.240	tr	0.0	0.0	0.0	tr	0.2	0.1	0.2	0.3
14:0	0.351	1.0	1.7	2.7	1.6	1.6	1.1	1.4	0.9	25.3
14:1	0.390	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
15:0	0.426	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.9	0.0
14:2	0.442	0.2	0.3	0.5	0.5	0.2	0.3	0.2		0.4
16:0	0.571	22.6	23.3	27.0	30.0	21.6	25.5	21.1	22.3	28.3
16:1	0.662	7.3	4.8	11.1	2.6	8.6	3.7	8.4	1.7	2.3
17:0	0.727	1.7	0.6	1.9	0.7	1.8	0.8	0.9	0.6	0.0
16:2	0.864	0.0	0.3	0.9	0.7	0.8	1.1	0.4	0.0	0.3
18:0	1.00	6.1	7.5	6.7	9.3	7.0	10.9	8.7	12.8	2.1
18:1	1.16	29.5	26.2	21.7	12.4	32.5	18.4	36.0	25.3	0.5
19:0	1.47	0.1	1.0	0.2	0.6	0.0	0.7	0.9	1.2	0.0
18:2	1.40	0.2			0.3	0.4	0.1	0.4	0.4	32.4
20:0	1.84	0.1	0.0	tr	0.0	0.0	0.0	0.0	0.0	0.0
18:3	1.90	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	4.4
20:1	1.99	1.1	0.0	1.0	0.7	0.6	0.8	0.8	0.8	0.0
18:4	2.21		0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0
20:2	2.35	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
21:0	2.44		0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
20:3	2.80	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4
20:4	3:46	2.0	5.0	1.7	4.6	1.5	3.5	1.4	2.8	0.0
22:1	3.78	1.4	0.0	2.0	0.0	0.8	0.0	0.3	tr	tr
20:5	4.42	14.3	7.6	9.8	8.9	14.0	7.8	9.4	7.9	0.0
22:3	5.45	1.1	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0
22:4	6.73	0.1	2.0	0.0	tr	0.0	0.0	0.0	1.2	0.0
22:5	8.02	0.9	0.7	2.0	3.6	1.1	1.6	0.9	1.3	0.0
22:6	9.55	10.0	18.8	9.6	23.1	7.4	22.7	8.1	19.2	0.0

* Carbon chain: double-bond number.

TABLE 3. FATTY ACIDS IN MITOCHONDRIA (MOLE %)*

Fatty Acid†	Average Retention Volume	Pelican Liver	Pelican Heart	Pelican Kidney	Cormorant Liver	Cormorant Heart	Grebe Liver	Grebe Heart	Murre Liver	Scoter Liver	Bass Liver	Sturgeon Liver	Flounder Liver	Seal Liver	Seal Heart
14:0 Br –16:0 Br	0.296 –0.502	0.8	3.5	2.9	1.2	2.5	0.9	2.6	1.2	1.0	2.9	2.1	4.2	1.1	4.0
16:0	0.571	17.0	17.3	13.1	20.2	11.3	16.3	12.8	15.6	17.2	19.2	18.8	19.2	14.5	10.2
16:1	0.656	4.9	6.5	6.3	4.9	3.3	4.4	4.8	4.3	4.5	10.6	6.0	11.9	3.9	2.8
17:0 Br	0.650	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	2.1	0.0	0.0
17:0 –16:3	0.743 –0.990	0.6	1.6	1.4	1.2	0.3	1.0	1.8	1.3	3.4	3.0	2.9	1.3	2.4	1.7
18:0	1.00	19.6	14.5	18.7	19.5	16.9	19.6	16.4	22.2	18.3	3.7	2.7	2.0	21.2	15.1
18:1	1.12	19.6	22.8	18.3	16.7	19.5	15.5	15.2	15.5	18.8	22.6	23.8	15.4	14.6	15.3
18:2	1.32	2.2	6.4	2.7	2.2	5.8	3.9	9.0	2.5	1.0	1.9	0.0	0.0	2.0	8.5
19:0	1.27	0.3	0.7	0.3	0.0	0.0	0.0	0.5	0.4	0.4	0.5	0.0	0.2	0.5	0.2
19:1 –21:1	1.46 –2.87	0.6	4.0	1.4	1.0	tr	1.3	0.8	1.0	1.8	5.5	12.4	3.5	1.0	1.5
20:0 18:3	1.68	0.0	1.0	0.5	0.3	0.8	3.9	3.4	tr	0.0	0.4	0.0	0.0	0.0	1.4
20:4	2.85	8.3	3.8	10.3	12.2	14.1	23.0	24.2	11.7	17.8	2.1	3.5	4.6	14.3	14.1
20:5	3.61	14.2	14.7	19.3	6.8	11.7	3.4	5.8	9.3	10.2	10.0	7.2	9.9	10.2	16.4
22:3	4.78	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	1.9	0.0	0.0
22:4	5.53	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4
22:5	6.14	0.0	0.0	0.8	0.5	0.0	1.8	0.0	2.4	0.6	1.9	2.3	3.8	3.4	1.4
22:6	6.97	11.9	2.4	4.0	12.7	13.3	5.0	2.9	12.6	5.0	15.2	15.3	20.0	10.7	6.0

* Minor and unknown peaks are combined. Complete analyses available from author's laboratory upon request.
† Carbon chain: double-bond number.

include the following: a wide variety of animals is available, they have been eating fish for generations, and collecting is less expensive and time consuming than prolonged feeding experiments. If there were no selectivity in the processes reponsible for forming the various structures within the bodies of these homoiothermic animals, their mitochondrial fatty acids should parallel those of marine oils in general; they should have a high content of polyunsaturated fatty acids of the linolenate type and a much lower dienoic-tetraenoic acid content (20). However, the relatively high content of dienoic and tetraenoic acids in the mitochondria of the birds and seals suggests that they may concentrate EFA by selective incorporation into the mitochondria.

TABLE 4. FATTY ACIDS IN MITOCHONDRIA (MOLE %)*

Fatty Acid	Fish	Marine Birds	Seal
18:0	2.8 ± 0.7	18.4 ± 1.7	18.2 ± 3.0
18:2	0.6 ± 0.4	4.0 ± 2.2	5.3 ± 3.2
20:4	3.4 ± 0.9	13.9 ± 5.2	14.2 ± 0.1
20:5	9.0 ± 1.2	10.6 ± 3.7	13.3 ± 3.1
22:6	16.8 ± 2.1	7.8 ± 4.3	8.4 ± 2.3

* Calculated from data in Table 3.

There is presumably a biochemical unity among mitochondria from various sources in order that they may carry out analogous metabolic reactions. As a result of this apparent unity, it may be possible to infer certain functions of the fatty acids in the context of their mitochondrial source and fatty acid type and distribution. For example, analyses presented in this paper and in a previous paper (7) show that, in fish mitochondria at least, there is no detectable linoleic or linolenic acids. No animal mitochondria have been shown to be devoid of arachidonic acid, although fish mitochondria are very low in this acid. On the other hand, sweet potato mitochondria, which carry out metabolic functions analogous to animal mitochondria (8), contain no detectable amounts of arachidonic acid but very high levels of linoleic acid. It may be possible to infer from this that there is not a specific requirement for either one of these acids to permit normal mitochondrial metabolism but, instead, there is a more general requirement for *some* polyunsaturated fatty acids.

In the mitochondrial membrane, the fatty acid residues would exist primarily as phospholipids (24), presumably in a lipoprotein matrix. In view of the effectiveness of surface active agents for fragmenting mitochondria (25), secondary valency forces, exemplified by those found in crystalline fatty acids or their methyl esters, are apparently important in maintaining the integrity of mitochondrial membranes. As a result, the physical behavior of the membranes should be to some extent a function of the physical properties of the phospholipid fatty acid residues as determined by their geometry, degree of unsaturation, and double-bond position. In general, the degree of unsaturation of the fatty acids of the mitochondria of birds and mammals that feed on fish closely parallels that of the fatty acids of fish mitochondria, averaging about 2 double-bond index units. However, analyses of the fatty acids of rat liver, beef heart, and chicken liver mitochondria (7) show them to be somewhat more saturated. Fish might require highly unsaturated fatty acids because their membranes and fat transport mechanism must function at temperatures closely approximating that of their environment. The physical characteristics of a more unsaturated fatty acid would obviously be desirable under these conditions.

The position of the double bonds of the fatty acids might also be considered as a factor affecting membrane behavior. This is reasonable because the position of the double bonds greatly affects the physical properties of the fatty acids, particularly those of *cis-cis* geometry (26). As the double bonds of the fatty acids are moved toward the methyl end, the melting point decreases markedly (27) and monomolecular films of these fatty acids become less stable (28) or more easily expanded, indicating a lesser degree of interaction between molecules and a more dynamic type of membrane system. Consequently, the highly unsaturated fatty acid residues of the linolenate family should contribute to a more flexible, less stable membrane system. The ease of hemolysis of fish erythrocytes compared to mammalian erythrocytes (29) and the faster initial rate of swelling of fish liver mitochondria as compared to rat liver mitochondria (30) lend support to this thesis.

TABLE 5. CLASSES OF MITOCHONDRIAL FATTY ACIDS*

Mitochondria	Fatty Acids (Mole %) Saturated	Mono-unsaturated	Poly-unsaturated
Marine Bird	37 ± 3	24 ± 3	39 ± 4
Fish	28 ± 1	38 ± 4	34 ± 4
Mammals (seal and rat)†	38 ± 4	18 ± 3	44 ± 4
Sweet potato	59	3	38

* Calculated from data in Table 3.
† Calculated from data of Richardson et al. (7).

TABLE 6. AVERAGE RATIO LINOLEATE FAMILY TO LINOLENATE FAMILY OF FATTY ACIDS IN MITOCHONDRIA*

Mitochondria	Mole Ratio†
Fish	0.1
Marine birds	0.6
Seal	0.8
Rat liver	>205‡
Beef heart	>500‡
Chicken liver	9‡

* Calculated from data in Table 3.
† Linoleate/linolenate families.
‡ Calculated from data of Richardson et al. (7).

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