Linolenic Acid Deficiency

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ABSTRACT

Linolenic acid deficiency has not been demonstrated clearly in warm blooded animals, yet circumstantial evidence suggests that n-3 fatty acids may have functions in these animals. The fact that several species of fish definitely require dietary n-3 fatty acids indicates that n-3 fatty acids have important and specific functions in these animals and suggests that such functions may also be present in warm blooded animals. It is also true that n-3 fatty acid distribution in tissues of birds and mammals appears to be under strict metabolic control, and that this complex metabolic control mechanism apparently has survived evolutionary pressure for a very long time. So far, attempts to produce linolenic acid deficiency in mammals have not revealed an absolute requirement for n-3 fatty acids. If functions for n-3 fatty acids do exist in warm blooded animals, it seems probable that they may be located in the cerebral cortex or in the retina, because these tissues normally contain high concentrations of n-3 fatty acids.

INTRODUCTION

Recently, committees of the WHO-FAO and of the American National Research Council have recommended that the human diet contain linolenic acid, in addition to specific recommendations for amounts of linoleic acid, which is known to be required in human beings. There is little direct evidence that linolenic acid is required in man or other warm blooded animals. This lack of evidence suggests that a requirement for linolenic acid, if any, is so low that almost any diet can provide the needed amount, at least in the species of animals that have been investigated in detail. It is certainly true that symptoms of linolenic acid deficiency in warm blooded species of animals are not well known or widely recognized. Despite the lack of evidence for essentiality of linolenic acid in the diets of human beings and other animals, many researchers feel that there is a function for this acid and its metabolites. Therefore, we have reviewed some of the observations that suggest an essential role for n-3 fatty acids in mammals.

METABOLISM OF LONG CHAIN POLYUNSATURATED FATTY ACIDS

Linolenic acid (9,12,15-octadecatrienoic acid, 18:3n-3, or 18:3ω3) and linoleic acid (9,12-octadecadienoic acid, 18:2n-6 or 18:2ω6) are synthesized by plants but not by higher animals. In plants, 18:3n-3 is commonly found in the membrane lipids of the chloroplast, the plant photoreceptor. 18:2n-6 is usually concentrated in the lipids of seeds. Herbivores will, therefore, obtain both of these fatty acids in their diets. These fatty acids are absorbed during digestion and metabolized by the animal body (most species) according to the major paths of elongation and desaturation:

18:3n-3→18:4n-3→20:4n-3→20:5n-3→22:5n-3→22:6n-3

18:2n-6→18:3n-6→20:3n-6→20:4n-6→22:4n-6→22:5n-6.

The endogenously formed oleic acid (9-octadecenoic acid, 18:1n-9 or 18:1ω9) is also elongated and desaturated:

18:1n-9→18:2n-9→20:2n-9→20:3n-9→22:3n-9→22:4n-9.

All of these families of fatty acids are processed by the same enzyme system, which prefers the n-3 family to the n-6 family and has least preference for the n-9 family. Hence, n-9 fatty acids are desaturated and elongated to a significant extent only when the concentrations of n-3 and n-6 fatty acids are relatively low, as happens when animals are fed a fat-free diet.

In the n-3 family, the member found most abundantly in animal lipids is usually 22:6n-3 (4, 7, 10, 13, 16, 19-docosahexaenoic acid), especially in land animals. In marine animals or fish, 20:5n-3 concentrations may also be quite high. 22:6n-3 usually occurs in highest proportions in phospholipids, especially in ethanolamine phosphoglycerides (EPG) of warm blooded animals, but also is found in neutral lipids, especially of fish and shellfish. These long chain polyunsaturated n-3 fatty acids in animal lipids may be obtained preformed in the diet, or they may be formed by the animal itself from dietary 18:3n-3.

DISTRIBUTION AND DIETARY REQUIREMENT OF N-3 FATTY ACIDS IN SHELLFISH

The lipids of many fish and shellfish, including commercially important species, have been analyzed. In shellfish, the proportion of 20:5n-3 is usually greater than that of 22:6n-3. This is true of Alaska king crab (1), the North

Carolina blue crab (2), a South African marine crab (3), and a freshwater crab from Lesotho (3). The proportion of 20:5n-3 is greater than that of 22:6n-3 in a European and an American species of oyster (4) as well as in an oyster from New Zealand (5). Four species of scallops, however, have proportions of 22:6n-3 higher than those of 20:5n-3 (6,7). In several varieties of prawns, the lipids have higher proportions of 22:5n-3 than of 22:6n-3 (8-13). Shrimp, like prawns, often have more 20:5n-3 than 22:6n-3 (12,14). It is not clear whether these shellfish control metabolically the amounts of 20:5n-3 and 22:6n-3 in their tissue lipids, or whether the amounts found are due to the composition of their dietary fatty acids. The fact that the growth of prawns is improved by the addition of long chain polyunsaturated oils to the diet (10,11) suggests that prawns may require preformed long chain polyunsaturated fatty acids. Kanazawa et al. (15) have shown that either 18:2n-6 or 18:3n-3 in the diet of prawns produces far better growth than dietary 18:1n-9 does, and that the effect of 18:3n-3 on growth is greater than that of 18:2n-6. Pollack residual oil, which contains 11% 20:5n-3 and 2% 22:6n-3 (11) produces far better growth than do either 18:2n-6 or 18:3n-3 (15). These data suggest that, in prawns, both n-3 and n-6 fatty acids are required, and that prawns have a limited ability to elongate and desaturate 18:2n-6 and 18:3n-3. We are not aware of any conclusive evidence that proves a requirement for n-3 fatty acids in shellfish. The occurrence of these n-3 fatty acids in specific lipids implies that the placement of these fatty acids is under metabolic control and that this process is advantageous to the shellfish.

DISTRIBUTION AND DIETARY REQUIREMENT OF N-3 FATTY ACIDS IN FISH

Fatty acids in the lipids of many species of fish have been analyzed, and the lipids of freshwater fish often contain higher proportions of n-6 fatty acids than are found in marine fish (16-18). Most fish contain large proportions of both 20:5n-3 and 22:6n-3. Channel catfish (19) and four varieties of murrels (20), all of which are freshwater fish, have higher proportions of 22:6n-3 than of 20:5n-3. Of marine fish, menhaden (21), pilchard (22), mackerel (23), slender tuna (24), horse mackerel (25), herring (26), cod (27-29), sole, halibut and dogfish (29) all have higher proportions of 22:6n-3 than of 20:5n-3, either in total lipids or in phospholipids. In Chinook and Coho salmon, 22:6n-3 is higher than 20:5n-3 (30,31). In juvenile pink salmon captured at sea, there is more 22:6n-3 than 20:5n-3 (32). The sand launce (33), two species of hake (34), and the Chilean anchovy (35) all have higher levels of 22:6n-3 than of 20:5n-3. In contrast to these fish, the lipids of the capelin have more 20:5n-3 than 22:6n-3, and also contain large proportions of 22:1 and 20:1 (36,37). This fish is said to be an important source of food for fish harvested commercially in the North Atlantic. In the species mentioned above, it is not known whether fatty acids are incorporated unchanged from the dietary lipids or whether the fish modify the dietary fatty acids by elongation and desaturation.

The dietary requirements for essential fatty acids have been investigated in several species of fish. In rainbow trout a dietary n-3 fatty acid is definitely required (38-44), and n-6 fatty acids (18:2n-6) are of little value for normal growth (38,39,41). Long chain polyunsaturated fatty acids or 22:6n-3 are more effective than 18:3n-3 in stimulating growth (39,43,44). These data indicate that rainbow trout require n-3 fatty acids and can elongate and desaturate 18:3n-3 to 22:6n-3, but suggest that preformed 22:6n-3 is more effective in stimulating growth. Possibly, the rate of elongation and desaturation of 18:3n-3 to 22:6n-3 limits growth in these fish.

The turbot cannot elongate or desaturate either 18:3n-3 or 18:2n-6 (45), so that it is not surprising that dietary long chain n-3 fatty acids are required for good growth (46-48). The carp grows well if fed either 18:3n-3 or 18:2n-6, but cod-liver oil stimulates growth more than either 18:3n-3 or 18:2n-6 does (49,50). Red sea bream apparently can elongate n-3 or n-6 fatty acids, but grow best when fed oils containing long chain n-3 fatty acids (51-53). Thus, at least four species of fish require n-3 fatty acids, and at present it appears that 22:6n-3 will satisfy the requirement. The ability to elongate and desaturate 18:3n-3 varies with the species. This ability may depend upon the fatty acid available in the natural food supply of the fish. If the natural food supply contains an abundance of 22:6n-3, it is unnecessary for the predator to be able to elongate and desaturate 18:3n-3.

DISTRIBUTION OF N-3 FATTY ACIDS IN TISSUES OF WARM BLOODED ANIMALS

In warm blooded animals, 22:6n-3 is the most abundant member of the n-3 family, and it usually is most concentrated in the ethanolamine phosphoglycerides (EPG) and serine phosphoglycerides (SPG) of a given tissue. The lipids of warm blooded animals usually contain

relatively little 22:6n-3, except for marine mammals and birds whose diet of fish contains much n-3 fatty acid. The total fatty acids or phospholipids of most tissues contain less than 5% of this fatty acid. Thus, it is particularly noteworthy that certain organs or organelles do contain high proportions of 22:6n-3. The best known examples are the lipids of brain or cerebral cortex, retina, spermatozoa, and testis.

In chicks 22:6n-3 is the major polyunsaturated fatty acid in brain phospholipids (54), and 22:6n-3 accounts for 14% of brain total fatty acids in the house sparrow (55). In total fatty acid of mink brain, 22:6n-3 is the most abundant polyunsaturated fatty acid (56). In synaptosomal membranes of mouse brain, 22:6n-3 accounts for 14% of phospholipid fatty acids (57) and in mouse brain microsomes, the proportion of 22:6n-3 increases with the age of the mouse (58). In EPG and SPG from rat brain synaptic vesicles, 22:6n-3 accounts for 31% and 37%, respectively, of the fatty acids (59). The amount of 22:6n-3 in mitochondria of rat brain increases with the age of the rat and becomes the major polyunsaturated fatty acid in 90-day-old rats (60). In a study of lipids in many bovine organs, Gonzato and Toffano reported that the content of 22:6n-3 in phospholipids of cerebral cortex was second only to that in retina (61). In the cerebral cortex of two species of whale and one species of dolphin, 22:6n-3 is 17 to 19% of the total fatty acids in EPG (62-64). The concentration of 22:6n-3 in the cerebral cortex is higher in adult whales and dolphins than in fetuses of these species (65). In the cerebral cortex of the human brain, 22:6n-3 of EPG increases with age and at the age of about 80 years can be 34% of fatty acids in this lipid fraction (66). These data indicate that 22:6n-3 is located with great specificity in particular parts of the brain, and is concentrated in the ethanolamine phosphoglycerides rather than being evenly distributed among lipid classes.

The retina is another organ into which 22:6n-3 is selectively incorporated in many species of animals. Anderson and colleagues (67,68) have reported the fatty acid patterns of phospholipids in whole retinas of dog, pig, human, sheep, bovine and rabbit. In these species, 22 to 23% of the total fatty acid in EPG is 22:6n-3, with lesser proportions of 22:6n-3 in serine, choline (CPG), or inositol phosphoglycerides (IPG). Weiss and Graf (69,70) have shown that the 22:6n-3 content of rabbit retinal phospholipids is highest in rabbits born in the summer, and that the percentage of 22:6n-3 increases with the age of the rabbit, up to 60 days. In the bovine retina, the highest proportions of 22:6n-3 are located in phospholipids, especially EPG and SPG, of retinal rod outer segments (ROS) (71-76) with lower proportions in the phospholipids of mitochondria, microsomes, or nuclei (77). In the rat the total retina or retinal rod outer segments contain large proportions of 22:6n-3 (78-81). If rats are fed a fat-free diet or essential fatty acid-deficient diet for 10 weeks to 11 months (79,80), there is little change in the proportion of retinal 22:6n-3, although the content of 20:3n-9 increases. Evidently, the 22:6n-3 molecules are tenaciously retained in the rat, a species in which the outer segment is normally renewed ca. every 14 days. If rats are raised for two generations on diets that contain little n-3 fatty acid, the content of 22:6n-3 can be reduced to less than half the normal percentage (81,82). In rats fed a fat-free diet, the normal renewal of retinal rod outer segments is impaired (83), although electron micrographs of the rod outer segments show no abnormalities (84). In rats made diabetic with alloxan, the percentage of 22:6n-3 in the retina drops from an initial value of 35% of the total fatty acid to 25% after 116 days (78). The retinal rod outer segments of the frog *Rana pipiens* contain high proportions of 22:6n-3, i.e., 46% of total fatty acids in SPG and 51% in EPG (85). These data indicate that the retina, and especially the photoreceptor or ROS, is particularly enriched with 22:6n-3 in the vertebrate species so far analyzed.

Another tissue in which 22:6n-3 is often very high is the testis. In this organ the lipids usually contain a 22-carbon fatty acid as a major polyunsaturate, but the structure of the chain varies with the species of the animal. In humans (86-89) or bulls (90,91), 22:6n-3 is a major polyunsaturate in the phospholipid of the testis. In boar testis proportions of 22:5n-6 and 22:6n-3 are both high (90,92). In mouse, guinea pig, and hamster testis, there is somewhat more 22:5n-6 than 22:6n-3 (86). The testes of rats, dogs and rabbits contain mainly 22:5n-6 with little 22:6n-3 (86,93). In chicken testis the major polyunsaturated fatty acid is 22:4n-6 (86). Lipids of spermatozoa normally contain the same major polyunsaturate present in the testis of the same species, and the concentration of this polyunsaturate is usually higher in the spermatozoa. Bovine spermatozoa contain remarkably large proportions of 22:6n-3, especially in choline phosphoglycerides (94-96). 22:6n-3 is also the major polyunsaturated fatty acid in spermatozoa of the rhesus monkey (97), ram (98,99), and man (96,99). In the boar both 22:5n-6

and 22:6n-3 are very high in spermatozoa (96,100), while in the dog (101) and rabbit (96), 22:5n-6 is the main polyunsaturated fatty acid. In the chicken the main polyunsaturated fatty acid in spermatozoa is 22:4n-6 (101). These data indicate that 22:6n-3 is selectively incorporated into the lipids of the testis and spermatozoa of certain mammalian species, particularly those of man, monkey, bull, and ram. Considerable amounts of 22:6n-3 are also present in the boar, mouse, guinea pig, and hamster, but 22:6n-3 is a minor component in rat, rabbit, dog, and chicken.

EVIDENCE FOR FUNCTIONS OF N-3 FATTY ACIDS

In fish and shellfish, the presence of large amounts of n-3 fatty acid in the tissues suggests that n-3 fatty acid may serve metabolic functions in these species. The facts that rainbow trout definitely require either 18:3n-3 or 22:6n-3 for good growth, and that n-6 fatty acids are inadequate, indicate that n-3 fatty acids do have some function in this species. Very probably, n-3 fatty acids also have definite functions in carp, red sea bream, and turbot, because the growth of these species is increased by dietary polyunsaturated fatty acids. It is possible that prawns may also require n-3 fatty acids.

In warm blooded animals, 22:6n-3 is selectively incorporated into EPG or SPG, and these particular phospholipids are selectively concentrated in the outer segment of the retinal rod, the cerebral cortex and sometimes in the testis and spermatozoa of many animal species. The 22:6n-3 must be derived from the diet, either directly or indirectly via 18:3n-3, and must be transported, incorporated into particular phospholipids, and installed in a specific location in a particular organ. All these steps must have been subjected to evolutionary pressure for vast periods of time, and have survived to the present. These facts suggest that 22:6n-3 or other n-3 fatty acids may have metabolic functions in warm blooded animals as well as in fish.

Is there any evidence that n-3 fatty acids have specific functions in warm blooded animals? The evidence is scanty, partly because most of the attention has been devoted to the n-6 fatty acids which are easier to work with in many ways. Nevertheless, there is evidence that n-3 fatty acids may have unique functions in warm blooded animals. It is known that dietary linolenic acid will improve growth in essential fatty acid (EFA)-deficient rats, although it will not cure the other symptoms of EFA deficiency, particularly infertility and dermatitis. Bernsohn and Spitz (102) have reported that dietary 18:3n-3 but not 18:2n-6 will restore to normal the activity of 5′-nucleotidase in brain homogenates from EFA-deficient rats. Capuchin monkeys fed a purified diet containing corn oil as a source of EFA developed dermatitis and fatty livers, which were cured by dietary linseed oil (103). This report is puzzling because the lipids of the deficient animals still contained considerable n-3 fatty acid. Electroretinograms were obtained from rats given, for 40 days, fat-free diets or the same diets supplemented with 2% ethyl oleate, ethyl linoleate, or ethyl linolenate. Rats given linoleate had greater amplitudes of both the a and b waves than were found with those given oleate or fat-free diets, and those given linolenate had the highest amplitudes of all (104).

Perhaps the most convincing evidence for a possible function of n-3 fatty acids in mammals was reported by Lamptey and Walker (105). They fed rats, for two generations, purified adequate diets containing either 10% safflower oil (low linolenate, about 300 mg/kg diet) or 10% soy oil (high linolenate, about 8400 mg/kg diet). In these rats the difference between diets had no effect on food intake, growth, litter size, brain size, distribution of lipid phosphorus in brain lipids, or several other parameters. However, in male rats of the second generation, differences in physical activity were associated with the different diets. Probably the most interesting observation was the fact that the rats fed the high linolenate diet performed much better on a Y-maze discrimination test than the low linolenate rats did. For the first three consecutive days of testing, both groups of rats performed equally, but for the following four days, the high linolenate rats increased their percentages of correct responses, whereas those given the low linolenate diet did not improve. In the brain phospholipids of the low linolenate rats, the content of 22:6n-3 was only 10 to 20% of the control (high linolenate) value. The authors noted that this learning impairment may not have been due solely to changes in brain composition because visual function may also have been influenced by changes in retinal lipid composition, which was not measured in this experiment.

FATTY ACID COMPOSITIONS IN TISSUES OF LINOLENIC ACID-DEFICIENT RATS

We decided to produce a dietary linolenic acid deficiency in rats, in order to locate the organs in which the deficiency would have the

TABLE I

Polyunsaturated Fatty Acids in Tissues from Linolenic Acid-Deficient and Control Rats

Tissue	Lipid fraction	Weight percentages of total methyl esters									
	Fatty acid:	20:4n-6		22:4n-6		22:5n-6		22:5n-3		22:6n-3	
	Diet:	C[a]	D[a]	C	D	C	D	C	D	C	D
Muscle	EPG[a]	13.0	21.6[b]	1.3	5.2	1.6	24.6	4.9	0.23	36.1	1.5
Brain	EPG	11.8	13.8	5.5	7.7	0.97	20.4	0.33	0.02	23.7	1.8
Heart	EPG	23.3	27.3	1.8	3.8	2.1	21.1	2.7	0.04	23.3	0.72
Liver	EPG	25.0	31.8	0.58	1.6	1.2	14.1	1.4	0.04	15.6	0.71
Lung	EPG	33.3	36.2	8.6	11.8	1.2	7.1	3.8	0.11	6.1	0.26
Spleen	EPG	38.7	41.3	6.5	9.7	1.2	6.9	3.6	0.08	6.0	0.25
Gastrointestinal tract	EPG	26.7	30.0	4.0	6.0	0.83	5.4	1.7	<0.01	5.5	0.34
Kidney	EPG	42.8	45.2	0.92	1.5	0.59	4.5	0.5	0.03	4.4	0.37
Testis	EPG	23.0	24.7	3.2	3.7	27.9	31.9	0.23	0.10	2.5	0.13
Retina	TFA[a]	9.6	10.3	1.5	2.8	0.7	26.4	0.45	<0.01	33.7	5.1
Plasma	TFA	15.8	22.0	0.25	0.44	0.30	2.5	0.35	0.02	3.2	0.18
Erythrocyte	TFA	15.3	21.3	0.82	2.0	0.79	2.6	1.1	0.06	2.7	0.22
Adrenal	TFA	19.5	17.0	6.4	5.9	0.76	2.7	1.2	0.07	1.9	0.10
Ovary	TFA	4.5	4.7	2.2	3.6	0.27	2.0	0.70	0.03	1.6	0.17
Adipose	TFA	0.43	0.50	0.08	0.08	<0.01	0.25	<0.01	<0.01	0.07	<0.01

[a]Abbreviations: C, control diet; D, linolenic acid-deficient diet; EPG, ethanolamine phosphoglycerides; TFA, total fatty acids.

[b]Underlined values are significantly different from controls, $P < 0.05$ or less. Controls were paired with equal numbers of deficient rats of same age and sex, n=3 to 9.

most severe effect upon lipid composition. Rats were fed for two generations a diet in which the only source of lipid was methyl linoleate, 1.25% by weight. Control rats were given 1.0% methyl linoleate and 0.25% methyl linolenate. We analyzed lipids in animals from both generations, of both sexes, and of different ages. The organs analyzed included brain, heart, lung, kidney, liver, testis, gastrointestinal tract, spleen, muscle, retina, adrenal, ovary, adipose tissue, erythrocytes, and plasma. The lipids of the first nine organs were fractionated by thin layer chromatography, and fatty acids of EPG, CPG, and IPG + SPG were analyzed by gas liquid chromatography. In the other tissues, fatty acid distributions of total lipid extracts were measured. Procedures for handling of rats, preparation of diets, and analysis of lipids have been described earlier (106,107).

We found no effect of diet composition upon growth rates, litter sizes, organ weights, or distributions of lipid phosphorus. The main effect of the deficiency was to lower the proportion of 22:6n-3 in all of the tissues examined. In control rats the highest proportion of 22:6n-3 were found in phospholipids, especially EPG, of muscle, brain, heart, and liver, and in the total fatty acids of retina (Table I). In other tissues proportions of 22:6n-3 were much lower. In the deficient rats, 22:6n-3 was mainly replaced by 22:5n-6 and to a lesser extent by 22:4n-6 or 20:4n-6 (Table I). In liver, kidney, heart, muscle, and gastrointestinal tract of deficient rats, the proportions of 22:5n-6 were higher in females than in males (data not shown).

Retina, brain, and muscle seemed to retain 22:6n-3 more strongly than other tissues did, because first generation deficient rats had higher proportions of 22:6n-3 in lipids of these tissues than were found in second generation rats (data not shown). In other tissues there was little difference between the generations in proportions of 22:6n-3.

The quantity of dietary 18:3n-3 needed for survival in rats is obviously very low, and was ca. 40 mg/kg diet in our experiments. This is far less than the requirement for n-6 fatty acid, which is about 10 g/kg diet or more for most mammalian species. Fish (trout) require roughly 1 or 2% of n-3 fatty acid in the diet, which is similar to the mammalian requirement for n-6 fatty acid. If n-3 fatty acids do have essential functions in mammals, or in rats in particular, only small amounts of n-3 fatty acids are needed to fulfill these functions. One of the functions of n-6 fatty acids is to furnish precursors for the formation of prostaglandins, endoperoxides, thromboxanes and related compounds which have powerful biological activity. There is evidence that n-3 fatty acids also can give rise to prostaglandin derivatives that have activity in mammalian tissues (108-110). The physiological effects of prostaglandins and related compounds are produced by very low concentrations of these substances.

CONCLUSION

Are n-3 fatty acids required in the human diet? There appears to be no direct evidence related to this question. Human beings, in common with many other species of mammals, have high proportions of 22:6n-3 concentrated in certain phospholipids of the cerebral cortex, retina, and spermatozoa. The proportion of 22:6n-3 in human cerebral cortex increases with age, as is the case in rats, mice, and whales. No one knows what the optimal level of 22:6n-3 in any tissue may be, or if there is an optimal level.

Before the question of the essentiality of n-3 fatty acids in man can be answered, a function for these molecules must be detected and demonstrated, probably in experimental animals. The use of linolenic acid-deficient diets should allow changes in function to occur and to be measured. If functions can be detected and measured in experimental animals, then procedures can be developed for measuring these functions in human beings. At present, it seems most probable that the functions of n-3 fatty acids will be located in retina or brain, and possibly in muscle or spermatozoa of certain species.

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