The Effects of Dietary α-Linolenic Acid on the Composition of Nerve Membranes, Enzymatic Activity, Amplitude of Electrophysiological Parameters, Resistance to Poisons and Performance of Learning Tasks in Rats[1]

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ABSTRACT Feeding rats diets containing oils that have a low α-linolenic acid [18:3(n-3)] content, such as sunflower oil, results in reduced amounts of docosahexaenoic acid [22:6(n-3)] and increased amounts of docosapentaenoic acid [22:5(n-6)] in all brain cells and organelles compared to rats fed a diet containing soybean oil or rapeseed oil. During the period of cerebral development there is a linear relationship between the n-3 fatty acid content of the brain and that of food until α-linolenic acid represents ~ 200 mg/100 g food [0.4% of the total dietary energy for 18:3(n-3)]. Beyond that point brain levels reach a plateau. Similar values are also found for other organs. The level of 22:6(n-3) in membranes is little affected by the dietary quantity of linoleic acid [18:2(n-6)] if 18:3(n-3) represents ~ 0.4% of energy. In membranes from rats fed diets containing sunflower oil, Na^+, K^+-ATPase activity in nerve terminals was 60%, 5'-nucleotidase in whole brain homogenate was 80%, and 2',3'-cyclic nucleotide 3'-phosphodiesterase was 88% of that in membranes from rats fed diets containing soybean oil. A diet low in α-linolenic acid leads to anomalies in the electroretinogram, which partially disappear with age. It has little effect on motor activity, but it seriously affects learning tasks as measured with the shuttle box test. Rats fed a diet low in α-linolenic acid showed an earlier mortality in response to an intraperitoneal injection of a neurotoxin, triethyltin, than did rats fed a normal soybean oil diet. *J. Nutr.* 119: 1880–1892, 1989.

INDEXING KEY WORDS:

- *α-linolenic acid* • *linoleic acid*
- *polyunsaturated fatty acids* • *brain*
- *peripheral nerve* • *myelin*
- *synaptosomes* • *5'-nucleotidase* • *liver*
- *organs* • *nutrition* • *rats*

The nervous system is the organ with the second greatest concentration of lipids, immediately after adipose tissue. These lipids are practically all structural and are not related to energy metabolism; they participate directly in the functioning of cerebral membranes. Cerebral development is genetically programmed: if one stage is missed or perturbed, the chances of recuperation are greatly reduced. Moreover, the renewal of neurons and oligodendrocytes is nil (a cell that disappears is not replaced), and the renewal of membranes is often very slow. It is therefore necessary to ensure that brain cells receive an adequate supply of nutrients, especially of lipids, during their differentiation and multiplication.

The dietary importance of polyunsaturated fatty acids is well known. These compounds contribute to a decreased incidence of cardiovascular disorders, and they are the precursors of biologically active derivatives. Nevertheless, the study of their structural role in nerve membranes, where they are qualitatively and quantitatively very important, has been neglected (1).

It is well known that dietary polyunsaturated fatty acids influence the fatty acid composition of the membranes (2), and that they are particularly important for normal cerebral development (3). In the nervous system, on average, one fatty acid out of three is polyunsaturated; these fatty acids participate in the structure of phospholipids but not that of sphingolipids. Results

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demonstrating the influence of polyunsaturated fatty acids on the structure and function of the nervous system are thus numerous (4–16). The authors have generally used diets simultaneously deficient in fatty acids of the n-6 and n-3 series. However, polyunsaturated fatty acids of the n-3 series have a very specific role in the membranes, especially in the nervous system, and all the cells and cerebral organelles are extremely rich in them (17).

We have previously shown that α-linolenic acid [18:3(n-3)] deficiency alters dramatically the fatty acid composition of various organs, including brain. Moreover, the speed of recuperation from these anomalies when a normal diet is fed is extremely slow for brain cells (18), organelles (19), brain microvessel (20) and peripheral nervous system (21), in contrast with other organs (18). Thus, we were interested in measuring some biochemical, electrophysiological, comportmental and toxicological parameters in rats fed an α-linolenic acid–deficient diet, and we have tentatively determined the α-linolenic acid requirement for developing brain and various organs in rats.

MATERIALS AND METHODS

Animals. Female Wistar rats originating from IFFA Credo (L'Arbresle, France) and bred in our laboratory were divided into two groups. One group was fed a purified diet containing 1.8% sunflower oil (940 mg of linoleic acid [18:2(n-6)]/100 g diet; 6 mg of α-linolenic acid [18:3(n-3)]/100 g diet). The other group was fed a diet containing 1.9% soybean oil [940 mg of 18:2(n-6)/100 g diet; 130 mg of 18:3(n-3)/100 g]. These two groups were fed their respective diet for two generations. The third and following generations were used for experiments. Because animals fed either diet ate similar amounts of food, they ate similar amounts of n-6 fatty acids. The diet composition and the fatty acid composition of dietary lipids were previously published (18). The diets were prepared in our laboratory.

For animals fed the sunflower oil diet, perinatal mortality has been found to be abnormally high (22). Three days after parturition, litters were adjusted to 10 animals. After weaning, animals were housed two per plastic cage; temperature and humidity were kept constant (21°C and 70%, respectively); and only males were used for experiments. Animals used for electroretinogram, learning performance and toxicology were not used for biochemical analysis.

In another series of experiments, 4 wk before mating females previously fed a diet containing sunflower oil were fed diets intermediate in α-linolenic acid content, which were obtained by adding increasing amounts of α-linolenic acid by addition of soybean oil to the sunflower oil diet (**Table 1, 2**). Diets intermediate in linoleic acid content were obtained by adding linseed oil and hydrogenated palm oil to rapeseed oil or sunflower oil (**Table 3, 4**). Thus, in these experiments α-linolenic acid content varied from 6 to 681 mg/100 g diet and linoleic acid from 150 to 6200 mg/100 g diet. Their pups were killed when 21 d old.

Separation of cells and organelles. Separation of neurons, oligodendrocytes, astrocytes, myelin, nerve endings (synaptosomes), mitochondria and endoplasmic reticulum (microsomes) has been previously described (17). The purity of fractions was evaluated by phase-contrast microscopy, enzyme marker assay, specific protein analysis (radioimmunoassay), electrophoresis and lipid analysis (17). Brain capillaries were prepared according to Goldstein (23) and their purity checked (24). Extraction methods for lipid fractions, their transmethylation and the analysis of methyl esters by capillary column gas chromatography have also been previously described (17) using the following conditions: Packard model 427 gas chromatograph (Chrompack-Packard, Les Ullis, France); glass capillary column; stationary phase FFAP; gas pressure vector H_2:0.6 bar; temperature: 190°C; detection by flame ionization.

Electroretinogram. Electroretinogram recordings were performed as previously published (25).

Learning behavior and motor activity. Learning behavior and motor activity were measured using open field and shuttle box tests in 60-d-old animals. Motor activity measurement was carried out in an individual box equipped with photoelectric cells that automatically recorded the movements of the animal (counts were made in 5-min periods for 1 h).

The open field test was performed in a space whose floor was divided into 25 squares with four different cylindrical objects at its center. For each minute, over a period of 5 min, three activity parameters were recorded: the number of rearings, the number of squares crossed (locomotor index), and the number of contacts with the objects (exploratory index). Two emotivity parameters (the number of defecations and the number of groomings) were also measured.

The shuttle box test is a test of conditioning, the purpose of which is to measure learning capacity. A cage was divided into two compartments with a connecting hatch; one had an electrifiable floor and a roof lamp. Ten seconds after the roof lamp lit, electric current was applied in the compartment occupied. The test lasted 15 min, and two shocks were delivered each minute. The conditioning of an animal was judged by the speed with which it passed from one compartment to the other after the lamp had lit in order to avoid the shock.

Each test period therefore gave three different types of result: the number of nonpassages (the animal having remained in the same compartment, despite the electric current, right up until the extinction of the light and termination of the electric shock); the number of passages with shock (the animal having reacted to the light signal but was unable to escape the shock for a more or less longer period of time); and the number of

TABLE 1

Total oil content and linoleic [18:2(n-6)] and α-linolenic [18:3(n-3)] acid composition of the 5% lipid diets containing various amounts of α-linolenic acid

Diet No.	Oil added		18:2(n-6)	18:3(n-3)	n-6/n-3
			mg/100 g diet		
I	Soybean	0			
	Sunflower	5,000			
	Total	5,000	3,102	9.4	330
II	Soybean	222			
	Sunflower	4,818			
	Total	5,040	3,102	23.6	131
III	Soybean	600			
	Sunflower	4,508			
	Total	5,108	3,102	48.0	65
IV	Soybean	990			
	Sunflower	4,188			
	Total	5,178	3,101	72.9	42
V	Soybean	1,391			
	Sunflower	3,860			
	Total	5,251	3,101	98.7	31
VI	Soybean	2,223			
	Sunflower	3,178			
	Total	5,401	3,100	152.0	20
VII	Soybean	3,105			
	Sunflower	2,454			
	Total	5,559	3,098	208.6	15
VIII	Soybean	4,053			
	Sunflower	1,677			
	Total	5,730	3,097	269.1	11
IX	Soybean	6,100			
	Sunflower	0			
	Total	6,100	3,096	401.0	8

TABLE 2

Fatty acid profile of the 5% lipid diets containing various amounts of α-linolenic acid [18:3(n-3)]

Fatty acid	Diet No. I	II	III	IV	V	VI	VII	VIII	IX
	mg/100 mg fatty acids								
14:0	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.3
16:0	6.4	6.8	6.9	7.4	7.7	8.2	9.1	9.5	11.2
16:1(n-7)	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.1
18:0	3.8	3.9	3.8	4.0	3.9	3.8	3.8	3.8	3.8
18:1(n-9)	22.4	22.5	22.5	22.8	22.8	22.7	22.4	21.2	21.0
18:2(n-6)	65.4	64.5	64.0	62.6	61.9	60.5	63.0	56.8	53.4
18:3(n-3)	0.2	0.5	1.0	1.5	2.0	3.0	4.0	5.1	7.0
20:0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.4
20:1(n-9)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:1(n-7)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
22:0	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.6	0.5
24:0	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Saturated	11.5	12.1	12.1	12.8	12.9	13.3	14.1	14.9	16.4
Monounsaturated	22.9	22.9	22.9	23.1	23.2	23.2	22.9	23.2	23.2
Polyunsaturated	65.6	65.0	65.0	64.1	63.9	63.5	67.0	61.9	60.4
n-6	65.4	64.5	64.0	62.6	61.9	60.5	63.0	56.8	53.4
n-3	0.2	0.5	1.0	1.5	2.0	3.0	4.0	5.1	7.0
n-6/n-3	327.0	129.0	64.0	42.0	31.0	20.0	16.0	11.0	7.6

TABLE 3

Amount of oil used to formulate diets containing various amounts of linoleic acid

n-6/n-3	n-3 = 300 mg/100 g		n-3 = 150 mg/100 g	
		g/100 g lipid		
1	Rapeseed	25.40	Rapeseed	9.48
	Linseed	7.38	Linseed	4.10
	Hydrogenated palm oil	67.22	Hydrogenated palm oil	86.42
2	Rapeseed	66.80	Rapeseed	30.64
	Linseed	2.06	Linseed	1.38
	Hydrogenated palm oil	31.14	Hydrogenated palm oil	67.98
4	Rapeseed	56.03	Rapeseed	41.32
	Soybean	27.25	Sunflower	7.30
	Hydrogenated palm oil	16.67	Hydrogenated palm oil	51.38
6	Rapeseed	19.02	Rapeseed	41.08
	Soybean	64.78	Sunflower	17.18
	Hydrogenated palm oil	16.20	Hydrogenated palm oil	41.74
8	Soybean	83.70	Rapeseed	40.72
	Sunflower	9.62	Sunflower	27.16
	Hydrogenated palm oil	6.68	Hydrogenated palm oil	32.12
10	Linseed	9.76	Rapeseed	40.48
	Sunflower	90.24	Sunflower	37.36
			Hydrogenated palm oil	22.16

TABLE 4

Fatty acid composition of diets containing various amounts of linoleic acid

	n-6/n-3[1]						n-6/n-3[2]					
Fatty acid	1	2	4	6	8	10	1	2	4	6	8	10
						mg/100 mg fatty acids						
14:0	1.1	0.5	0.3	0.3	0.2	0.1	1.0	1.0	0.7	0.6	0.5	0.4
16:0	27.5	16.5	13.0	14.1	11.5	6.1	39.9	33.3	25.6	21.5	17.5	14.4
18:0	8.1	6.0	4.8	5.2	4.4	3.8	14.9	10.5	9.6	8.3	7.0	5.9
18:1(n-9)	48.2	57.4	46.2	31.8	23.2	21.3	37.7	39.8	47.4	46.2	44.2	42.7
18:1(n-7)	1.3	1.1	3.4	2.6	1.7	1.4	0.9	0.9	0.8	1.0	1.1	0.8
18:2(n-6)	6.7	12.6	25.0	38.8	51.7	60.6	3.0	6.9	12.7	19.3	26.2	32.1
18:3(n-3)	6.6	5.8	6.1	6.3	6.5	5.9	3.0	3.6	3.1	3.1	3.2	3.3
20:0	0.3	0.4	0.4	0.4	0.3	0.2	0.4	0.3	0.4	0.4	0.4	0.4
20:1(n-7) + (n-9)	0.2	0.6	0.6	0.3	0.2	0.1	0.1	0.3	0.4	0.4	0.4	0.5
22:0	0.1	0.2	0.2	0.2	0.3	0.5	0.1	0.1	0.1	0.2	0.2	0.3
n-6/n-3	1.01	2.17	4.10	6.16	7.95	10.27	1.0	1.92	4.10	6.22	8.19	9.73
Lipids, %	5.4	5.7	5.1	5.3	5.3	5.3	5.2	5.2	5.2	5.2	5.0	5.1

[1]n-3 = 300 mg.
[2]n-3 = 150 mg.

passages without shock, which was the number of avoidances (the animal having made the connection between the light and the shock and was thus able to avoid it).

All these tests were carried out on animals that had never been previously tested. The cages were cleaned after each utilization.

Resistance to the neurotoxic agent triethyltin. Triethyltin (Sigma Chemical, St. Louis, MO) was made up in physiologic solution and administered at a dosage of 0.5 ml/100 g body weight. The product was administered by the intraperitoneal route in one single dose to 60-d-old animals, which had been fasted for 16 h and had been under observation for 8 d. Feeding was resumed 4 h after administration of the product. Behavior, appearance of the animals, side effects and mortality were noted daily for 14 d after the injection of triethyltin.

Determination of the lethal dose (LD_{50}) was made after establishment of the LD_0 and LD_{100} and of intermediate doses chosen in geometric progression. The LD_{50} was calculated using a linear regression model.

Measurement of enzyme activity. Enzyme activity was determined as previously described for Na^+, K^+-

ATPase (EC 3.6.1.3), 5'-nucleotidase (EC 3.1.3.5) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, EC 3.1.4.37) (26).

Statistics. Statistical analysis as performed using Student's *t*-test.

RESULTS AND DISCUSSION

Body weight, food intake and growth were similar in rats fed either sunflower oil or soybean oil. For 21-d-old animals, body weight was 48.2 ± 3.8 and 45.6 ± 3.2 g for animals fed sunflower or soybean oil, respectively; body weight was 278.0 ± 7.5 and 297.2 ± 10.0 g for 60-d-old animals, respectively. With both diets, even with low levels of lipid, essential fatty acid deficiency was not detected, no dermatological signs were observed, and eicosatrienoic acid [20:3(n-9)] was absent in tissues or organelles. This was expected, since 1.5% sunflower oil in the diet provides ~ 1.8% of energy as 18:2(n-6).

Composition of cells and organelles in the nervous system and in other organs in rats fed diets containing sunflower oil or soybean oil. Total amount of polyunsaturated fatty acids in cells and intracellular organelles in the two dietary groups was not different. In animals fed the sunflower diet, cells and organelles contained relatively less cervonic acid [docosahexaenoic acid; 22:6(n-3)] and more docosapentaenoic acid [22:5(n-6)] than did animals fed the soybean diet. If 60-d-old animals fed the sunflower diet are compared with those fed the soybean oil diet, the total n-3/n-6 ratio was 6% of the control in oligodendrocytes, 8% in myelin, 50% in neurons, 17% in nerve endings (synaptosomes), 33% in astrocytes, 14% in mitochondria and 20% in microsomes. On the other hand, the saturated and monounsaturated fatty acids were not different between the two groups. Specific alterations in 22:6(n-3) and 22:5(n-6) are shown in **Table 5**. The membranes of cerebral cells and organelles were as deficient as those of other organs. However, there was marked conservation of dietary α-linolenic acid (and a reutilization of its very-long-chain derivatives), because if its quantity is 4% of the control in the diet, it only results in, at the worst, a 90% decrease in the fractions that we examined.

These results concerning n-3 fatty acids are in agreement with alterations we previously found in phosphatidylethanolamine in animals fed diets based on peanut or rapeseed oils (27, 28).

Definition of the minimum necessary amount of α-linolenic acid. When diets intermediate in α-linolenic acid content were fed, increasing quantities of dietary 18:3(n-3) resulted in an overall increase of 22:6(n-3) (**Fig. 1**) and, inversely, a decrease of 22:5(n-6) (**Fig. 2**) in all tissues studied. In whole brain, retina, myelin, nerve endings and sciatic nerve the level of 22:6(n-3) increased linearly with a 18:3(n-3) intake from 0 to 200–250 mg/100 g of diet; it then reached a plateau [the inverse was observed for 22:5(n-6)]. In the liver and heart the response was rapid up to 200 mg of 18:3(n-3) per 100 g of diet, beyond which there was a slower increase. The slope of the curve changed at the same value in kidney and muscle. In adipose tissue the extremely low amount of 22:6(n-3) increased linearly with increasing α-linolenic acid in the diet over the entire range of 18:3(n-3) fed (25–481 mg/100 g diet).

The α-linolenic acid requirement increased slightly with the dietary content of linoleic acid. For the developing brain in the rat, for 1000, 2500 and 5000 mg 18:2(n-6)/100 g diet, requirements for 18:3(n-3) were ~ 175, 200 and 250 mg/100 g diet, respectively (**Fig. 3**).

TABLE 5

Levels of cervonic acid **[22:6(n-3)] and docosapentaenoic acid [22:5(n-6)] in cells and cerebral organelles of rats fed sunflower oil, with values expressed as a percentage of the respective values in rats fed soybean oil[1]**

	22:6(n-3)	22:5(n-6)
	%	%
Nervous system		
Neurons[2]	48 ± 5	214 ± 19
Nerve endings[2]	27 ± 4	1088 ± 115
Oligodendrocytes[2]	10 ± 2	240 ± 41
Myelin[2]	14 ± 2	1200 ± 192
Astrocytes[2]	47 ± 6	344 ± 60
Mitochondria[2]	25 ± 4	917 ± 102
Microsomes[2]	28 ± 2	592 ± 49
Retina[4]	36 ± 3	1280 ± 110
Brain capillaries[3]	26 ± 5	362 ± 46
Sciatic nerve[4]	28 ± 4	1000 ± 122
Others[5]		
Serum	30 ± 3	480 ± 42
Heart	24 ± 4	480 ± 51
Muscle	24 ± 3	330 ± 43
Lung	27 ± 4	407 ± 72
Testis	32 ± 3	131 ± 14
Liver[4]	38 ± 3	560 ± 52
Kidney	45 ± 5	530 ± 54

[1]Values are expressed as mean percentage ± SD. In rats fed sunflower oil, 22:6(n-3) and 22:5(n-6) levels of all organs, cells and organelles examined were significantly different ($P < 0.01$) from those of rats fed soybean oil when the *n* used for statistical analysis was the number of litters. Absolute amounts of fatty acid were in good agreement with our previously published results (17–21).

[2]For neurons, astrocytes, oligodendrocytes, myelin, nerve endings, microsomes and mitochondria, each point represents the mean value from three different preparations. Each preparation required 16 animals, thus each individual point represents 48 animals (minimum of six different litters).

[3]For brain capillaries, each point represents the mean value for four different preparations. Each preparation required 14 animals, thus each point represents 56 animals (eight different litters).

[4]For sciatic nerves, retina and liver, each point represents the mean value from three different preparations. Each preparation required four animals, thus each point was obtained from 12 animals (six different litters).

[5]For serum and all organs except liver, each point represents the mean value obtained from 14 samples (one animal per sample). The 14 animals were from four different litters.

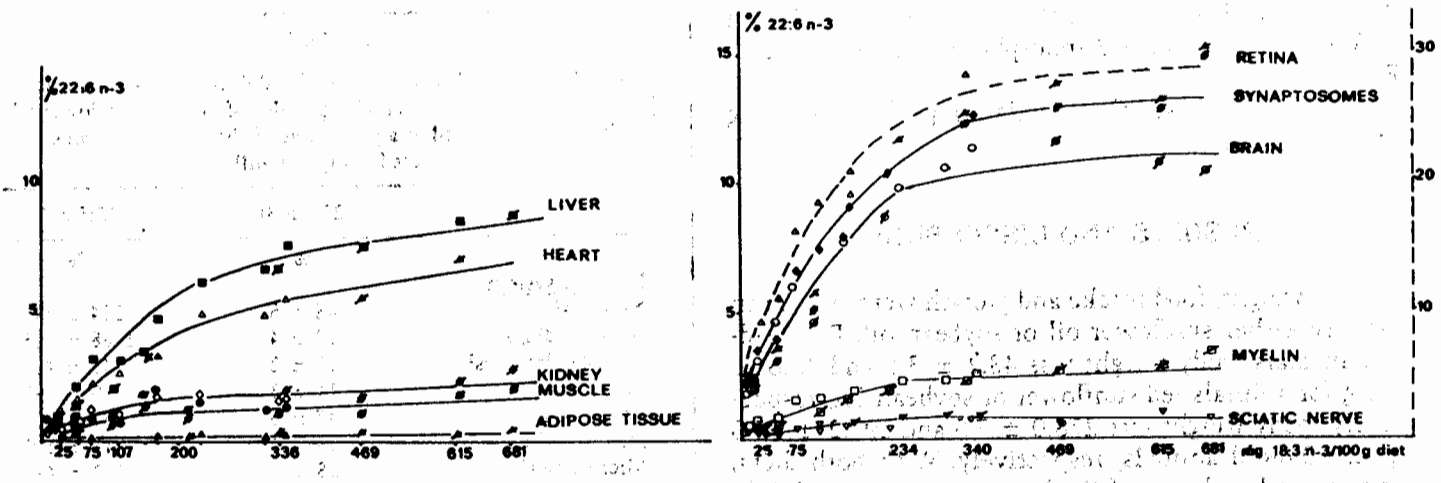

FIGURE 1 Relationship between dietary α-linolenic acid content and 22:6(n-3) levels in different organs and fractions. Rats were fed a diet containing 5 or 10% lipids (thus providing two levels of 18:2(n-6); 3200 mg/100 g diet, normal symbols; 6400 mg/100 g diet, symbols with a diagonal bar. Intermediate α-linolenic acid contents were obtained by increasing the quantity of sunflower oil and decreasing that of soybean oil. Data are means; SD is less than 15%. ***Left panel***: For liver, heart, kidney, muscle and adipose tissue, each point represents the mean value of at least five different preparations, and each preparation required at least three animals. Thus, each point represents at least 15 animals (from at least five different litters). Values with 681 mg of 18:3(n-3)/100 g differed significantly from those with 25 mg of 18:3(n-3)/100 g diet ($P < 0.001$ for liver and heart; $P < 0.01$ for kidney and muscle; $P < 0.05$ for adipose tissue). ***Right panel***: For brain, each point represents the mean value of at least five animals. For myelin and synaptosomes, each point represents the mean value of at least three different preparations, and each preparation required at least four animals. Thus, each point represents at least 12 animals (from at least three different litters). For neurons, astrocytes and oligodendrocytes, each point represents the mean value for at least four different preparations, and each preparation required at least 16 animals. Thus, each point represents at least 48 animals (at least six different litters). For brain capillaries, each point represents the mean value of at least four different preparations, and each preparation required at least 14 animals. Thus, each point represents at least 56 animals (at least eight different litters). For sciatic nerves and brain, each point represents the mean value of at least three different preparations, and each preparation required at least four animals. Thus, each point represents at least 12 animals (six different litters). Values with 681 mg of 18:3(n-3)/100 g differed significantly from those with 25 mg of 18:3(n-3) ($P < 0.001$ for brain, retina and synaptosomes; $P < 0.01$ for myelin; $P < 0.05$ for sciatic nerve).

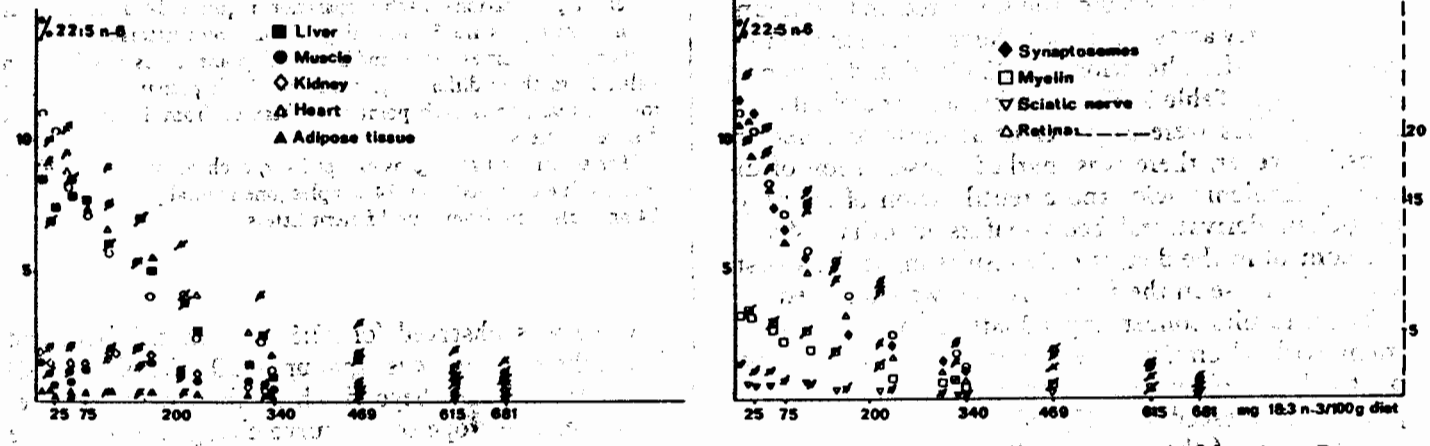

FIGURE 2 Relationship between dietary α-linolenic acid and 22:5(n-6) acid levels in different organs and fractions. Rats were fed a diet containing 5 or 10% lipids (thus providing two levels of 18:2(n-6)), 3200 mg/100 g diet, normal symbols; 6400 mg/100 g diet, symbols with a diagonal bar. Intermediate α-linolenic acid contents were obtained by increasing quantities of sunflower oil and decreasing that of soybean oil (see Materials and Methods). Data are means; SD is less than 15%. ***Left panel***: For liver, heart, kidney, muscle and adipose tissue, each point represents the mean value of at least five different preparations, and each preparation required at least three animals. Thus, each point represents at least 15 animals (from at least five different litters). ***Right panel***: For myelin and synaptosomes, each point represents the mean value of at least three different preparations, and each preparation required at least four animals. Thus, each point represents at least 12 animals (from at least three different litters). For retina, each point represents the mean value of at least three different preparations, and each preparation required at least six animals. Thus, each point represents at least 18 animals. For sciatic nerve, same remarks as in Fig. 1, *right panel* legend.

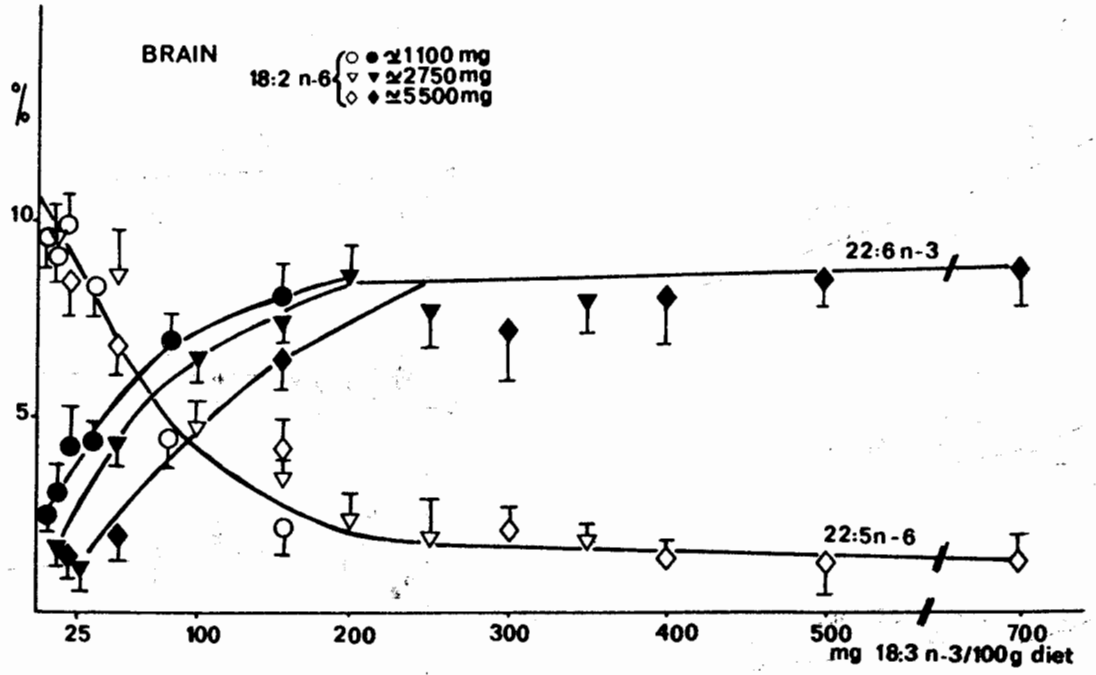

FIGURE 3 Brain cervonic acid concentration in relation to dietary content of linoleic acid. Each point represents the mean value of at least five different preparations, and each preparation required at least three animals. Thus, each point represents at least 15 animals from at least five different litters. The bars are the standard error of quadruplicate analyses.

Interestingly, increasing the amount of 18:2(n-6) in the diet from 150 mg to 6200 mg/100 g diet had little effect on the level of 22:6(n-3) in brain, sciatic nerve, myelin, synaptosomes and retina or in various organs, except in adipose tissue (Fig. 4). However, increasing the amount of 18:2(n-6) from 300 to 1400 mg/100 g diet (depending upon the organ) increased arachidonic acid [20:4(n-6)] content; after a dietary level of 1400 mg of 18:2(n-6) was reached, 20:4(n-6) content reached a plateau, except in adipose tissue (unpublished results). Higher quantities of dietary 18:2(n-6) only altered 22:5(n-6) and to a lesser extent 22:4(n-6). Thus, the levels of 22:6(n-3) and 20:4(n-6) are tightly controlled by some unknown mechanism: deficiency in n-3 fatty acids or excess of 18:2(n-6) both provoke only the accumulation of 22:5(n-6).

Direct uptake by the brain of 18:2(n-6) and 18:3(n-3) is probably small. These fatty acids, although possibly synthesized in the developing brain (9, 29), have to be desaturated and elongated in the liver to longer chains, which in fact could be the essential cerebral fatty acids as appears to have been established by cell culture techniques (30). The brain contains practically no linoleic or α-linolenic acid; cultured nerve cells cannot synthesize measurable amounts of docosahexaenoic acid [22:6(n-3)]. Only the addition of 20:4(n-6) and 22:6(n-3) to nerve cell culture medium results in, on the one hand, a better functioning of the neurons (measured, for example, by the renewed release of neurotransmitters) (31), and, on the other, the multiplication and differentiation of oligodendrocytes with membranes having a normal fatty acid composition (30).

A slight excess of dietary n-3 very-long-chain polyunsaturated fatty acids does not lead to an accumulation in the nervous system: in the brains of animals whose diet is enriched in n-3 fatty acid (5% lipid added with 1% menhaden oil), the vitamin E, conjugated dienes and malonaldehyde contents are not changed, and neither are the glutathione peroxidase, catalase and superoxide dismutase activities (32). Slightly excess amounts of fats are unlikely to be attacked by free radicals or to be broken down into toxic derivatives by peroxidation in the brain. However, feeding animals a large excess of fish oil (15% cod liver oil) alters the brain content of 22:6(n-3) and 20:5(n-3) (33).

Enzyme activities (Table 6). Na^+, K^+-ATPase (EC 3.6.1.3) activity in the synaptosomes (nerve endings) of animals fed a sunflower oil diet was one half of that in rats fed a soybean oil diet, who show normal levels of enzyme activity. Interestingly, a specific deficiency in α-linolenic acid produced a decrease in the activity of this enzyme, while a simultaneous deficiency in linoleic and α-linolenic acids resulted in an increase in activity (16). Na^+, K^+-ATPase controls the ionic flow resulting from nerve transmission, and it consumes half the energy used by the brain. (In an adult human this organ only represents 2% of body weight but consumes 20% of energy).

The activity of 5'-nucleotidase (EC 3.1.3.5) was decreased by 30% in total brain but not in myelin or in synaptosomes, which suggests that the enzyme level in cellular membranes is probably very altered. These results are in agreement with those of Bernsohn and Spitz (34), who showed that the decrease in the activity

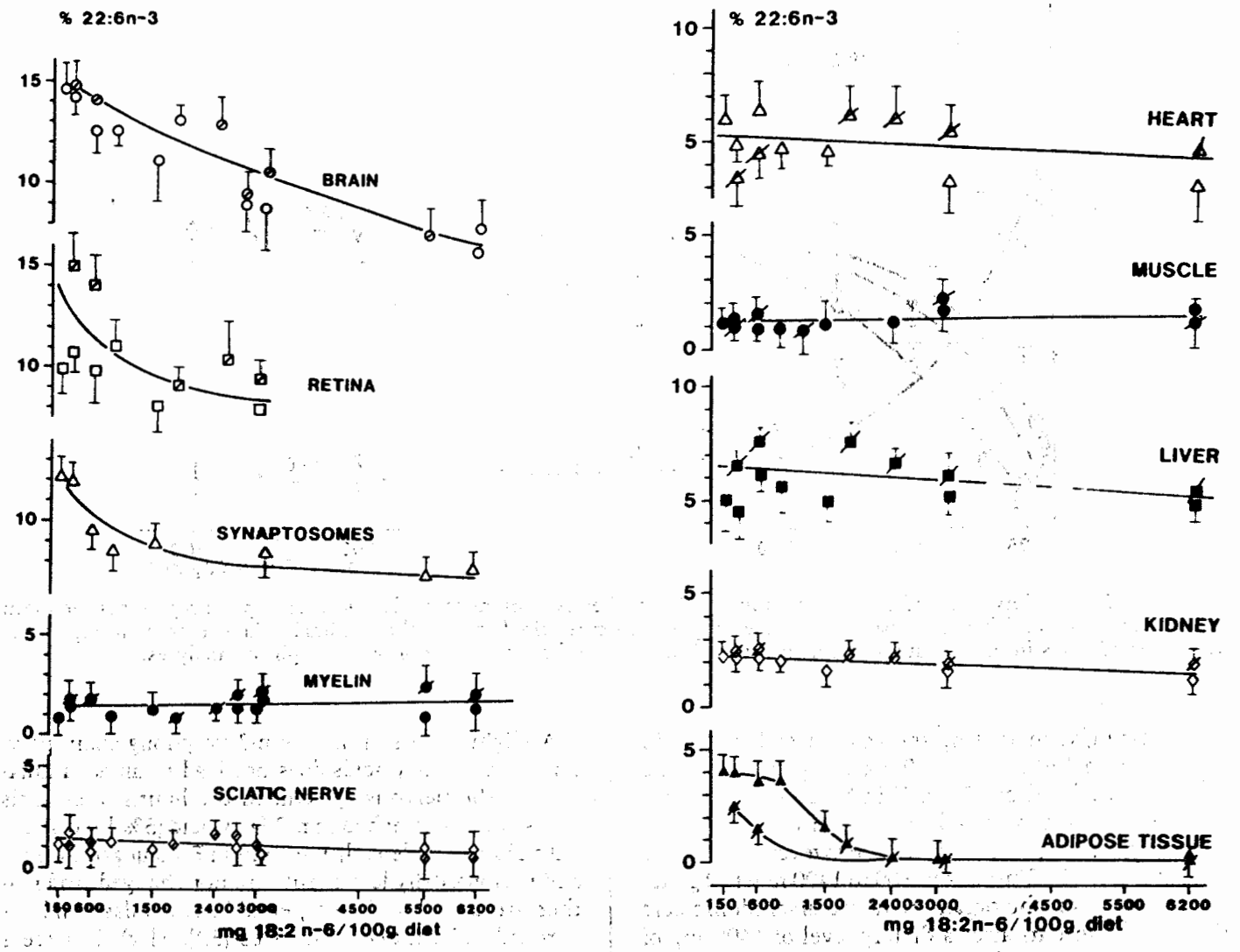

FIGURE 4 Relationship between dietary linoleic acid content and 22:6(n-3) level in organs. Rats were fed varying amounts of 18:2(n-6) (from 150 mg to 6200 mg/100 g diet); the amount of 18:3(n-3) was 150 mg for half the points (normal symbols), and 300 mg for the others (symbols with a diagonal bar). Number of animals for each point is the same as in Figure 1. Note that the scale for 18:2(n-6) is very large; in comparison, the one for 22:6(n-3) is very small. Thus, only a small percentage alteration in membrane 22:6(n-3) was seen when dietary 18:2(n-6) was increased by ~ 4100%. Except for adipose tissue, values for 22:6(n-3) when 150 mg of 18:3(n-3) was fed were not statistically different from those when 300 mg of 18:3(n-3)/kg diet was fed.

TABLE 6

Activities of membrane enzymes in brain from rats fed sunflower oil diet compared to those from rats fed soybean oil diet[1]

Enzyme[2]	Brain	Myelin	Nerve Endings
5'-Nucleotidase	0.70 ± 0.07[a]	0.94 ± 0.08	1.20 ± 0.01
Na^+, K^+-ATPase	0.95 ± 0.07	1.10 ± 0.09	0.55 ± 0.70[b]
CNPase	0.95 ± 0.10	0.78 ± 0.09[a]	ND

[1]Each value is expressed as the ratio of enzyme activity in sunflower oil–fed rats to that in soybean oil–fed rats ± SD; n = the mean of four preparations. Each preparation for myelin and nerve endings required 10 animals from at least three different litters; ND, not detected. Superscript letters denote a significant difference between sunflower oil – and soybean oil–fed rats at [a]$P < 0.01$ and [b]$P < 0.001$. Values obtained with soybean oil–fed animals compare favorably with our previous results in rat and mouse (26, 63).

[2]Na^+, K^+-ATPase (EC 3.6.1.3) activity was 90, 205 and 236 nmol Pi/(min · mg protein); 5'-nucleotidase (EC 3.1.3.5) activity was 26, 70 and 33 nmol Pi/(min · mg protein); and CNPase (EC 3.1.4.37) activity was 4, 19 and 0.5 nmol/(min · mg protein) in brain, myelin and nerve endings, respectively.

of the enzyme produced by a simultaneous deficiency of linoleic and α-linolenic acids is corrected only by the addition of α-linolenic acid to the diet.

CNPase (EC 3.1.4.37) activity in myelin was decreased by a deficiency in α-linolenic acid, even though this membrane is considered to have low metabolic activity.

The activity of acetylcholinesterase is also modulated by dietary lipids (35). In contrast, we found that some brain enzymes were not altered by α-linolenic acid deficiency: methyltransferase, phosphorylcholine citydyl transferase (P. Fonlupt, unpublished data), and AMP and CMP cytosolic cyclic nucleotide phosphodiesterase (A. F. Prigent, unpublished data) as well as binding of dihydro-alphenolol and diazepam (A. Fonlupt, unpublished data). The effects of the nature of membrane fatty acids on enzyme activities have been studied in numerous organs (6, 36), thus the brain does not escape the general rule.

Electroretinogram. The retina is one of the tissues that is richest in n-3 polyunsaturated fatty acids (5). Overall deficiencies in polyunsaturated fatty acids induce, in the long term, modifications in the distribution of membrane fatty acids in the retina (37), which are related to perturbations in the amplitude of the *a* and *b* waves in the electroretinogram (ERG) (38). But the direct influence of dietary fatty acids on the electroretinogram has been reported to depend on the species, age and the duration of the deficiency, and it has even been denied (39). Results (**Fig. 5**) reveal substantial perturbations in the ERG of animals deficient in α-linolenic acid. In 4-wk-old animals fed a sunflower oil diet the *a* and *b* waves were only detectable at stimulation intensities that were ten times those of animals fed a soybean oil diet.

At maximum stimulation intensity, in 4-wk-old rats fed sunflower oil there was a 45% decrease in the amplitude of wave *b* and a 38% decrease in the amplitude of wave *a* compared to animals fed soybean oil. In 6-w-old rats, the values were only 15% for wave *b* and 20% for wave *a*. Finally, in the adult, only the amplitude of wave *a* showed lower (20%) amplitude compared to controls.

These results are in agreement with our previous results that, in young rats (21 d) fed a peanut oil diet, the amplitude of the ERG is one third of that obtained with animals fed a rapeseed oil diet (40). Thus, there is no doubt that α-linolenic acid deficiency alters the electroretinogram in a number of species (41).

Learning test results. It is known that a deficiency of both linoleic and α-linolenic acids alters the learning capacity of animals (15, 42, 43).

Results for the motor activity and open field tests were nearly normal for rats fed the sunflower oil diet (data not shown). On the other hand, the learning behavior of these animals was very perturbed, as shown by the shuttle box test (**Fig. 6**). At the first test period it was clear that animals fed soybean oil made a quicker

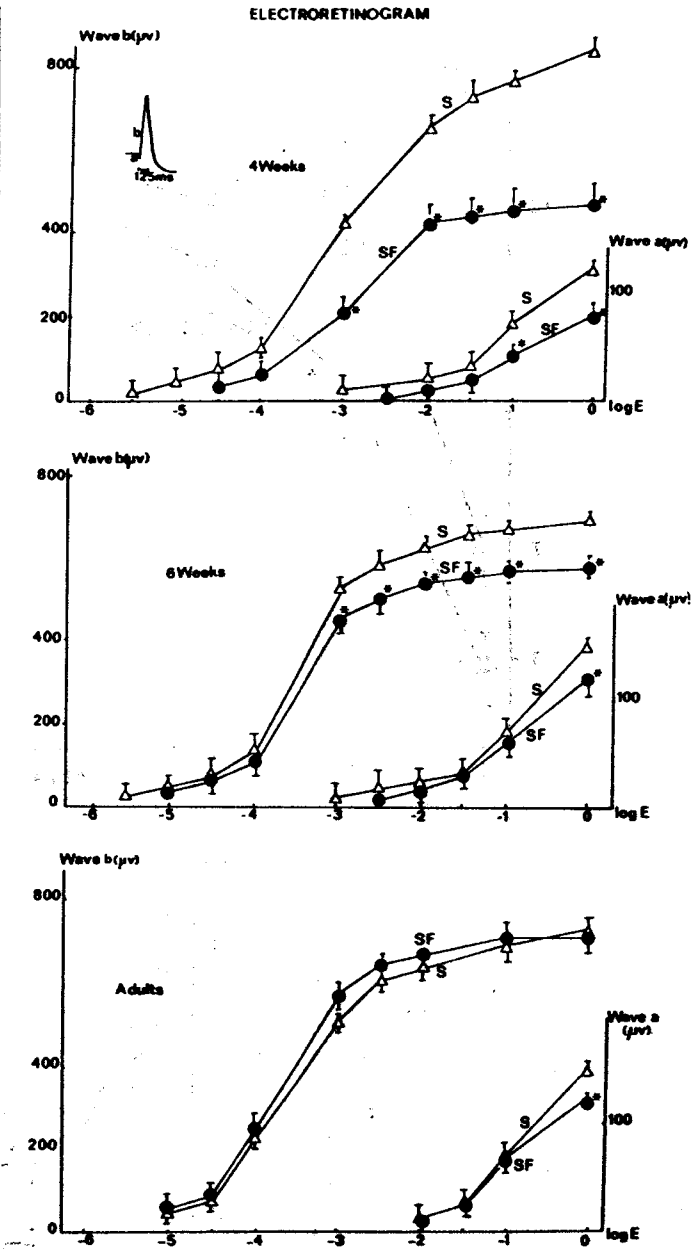

FIGURE 5 **Electroretinogram *a* and *b* waves in young rats fed sunflower oil diets or soybean oil diets. Each point is the mean of values from at least 12 60-d-old rats from three different litters. SF, sunflower oil diet; S, soybean oil diet; * significantly different ($P < 0.01$).**

association between the light signal and the electric shock, since on average they avoided seven shocks out of 30, while animals fed sunflower oil only avoided two. The number of passages with shock was the same in both groups, but the number of nonpassages was much higher in the sunflower oil–fed group. This means that many animals fed the sunflower oil diet underwent electric shock for the whole duration of the stimulus without attempting, or without being able, to escape. The animals fed the soybean oil diet averaged two nonpassages out of 30 stimulations, while the animals fed the sunflower oil diet averaged 10. As a function of the

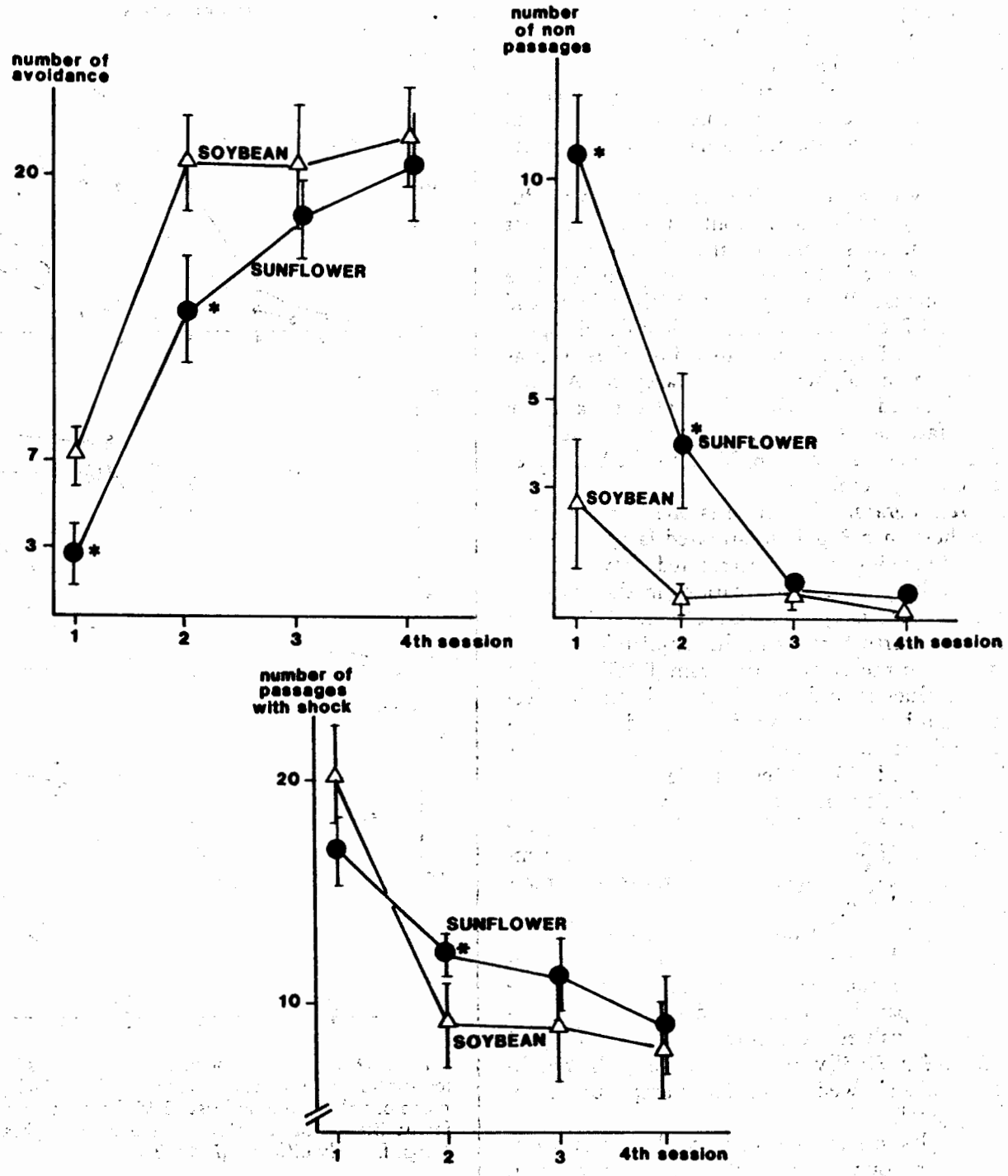

FIGURE 6 Results of learning tests (shuttle box) in animals fed sunflower oil diets or soybean oil diets. Each point is the mean of values from at least seven rats from three different litters. Animals were 60 d old; * significantly different ($P < 0.01$).

number of test periods, differences tended to decline and then disappeared at the third test period. Learning impairment in rats fed the sunflower oil diet could be related to a decrease in learning capacity. These results are in agreement with those of Lamptey and Walker (44) and Yamamoto et al. (45), using different techniques.

Interestingly, the extinction of learning was significantly longer in animal deficient in α-linolenic acid (46).

Mortality in animals tested with the neurotoxic agent triethyltin. The LD_{50} of animals fed the soybean oil or sunflower oil diets did not differ significantly (6.18 vs. 6.02 μl/kg, respectively). But the animals fed the sunflower oil diet died more rapidly than did those fed the soybean oil diet (**Fig. 7**). The nature of the polyunsaturated fatty acids could, therefore, affect the process of detoxification at the level of hepatic membranes (47).

The sensitivity to alcohol (ethanol) shown with di-

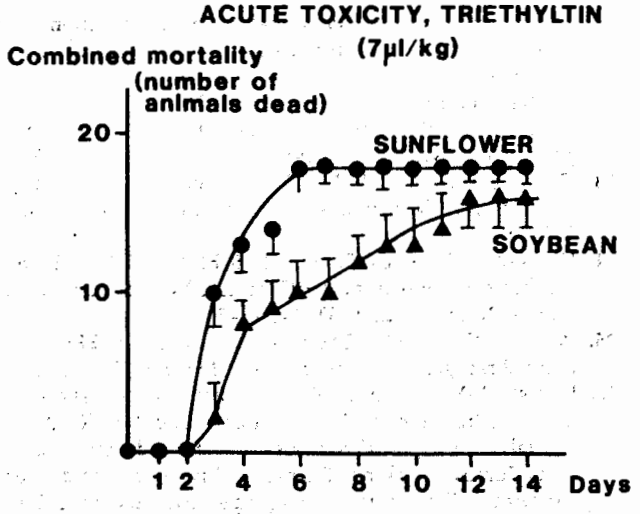

FIGURE 7 Effect of the neurotoxic agent triethyltin. The curves represent cumulative mortality as a function of time after acute intoxication with 7 µl triethyltin/kg body weight. This study was conducted with 50 sunflower oil–fed rats and 50 soybean oil–fed rats (60 d old). All points between 3 and 11 d were significantly different comparing animals fed sunflower oil and those fed soybean oil ($P < 0.01$).

phenylhexatriene (DPH) is also decreased significantly in animals fed sunflower oil (48). Concomitantly, rats fed sunflower oil are more sensitive to ethanol-induced hypothermia (48), illustrating the importance of diet to membrane sensitivity and animal response to ethanol, regardless of the exact mechanisms (48). In nerve-ending membranes, fluidity is affected by the diet, depending on the membrane region. Feeding the sunflower oil diet compared to the soybean oil diet results in less fluidity in the surface polar part of the membranes probed by TMA- or PROP-DPH but greater fluidity in the apolar part of the membranes (probed by DPH) (48).

Conclusions. A diet deficient in α-linolenic acid produces marked deviations in the polyunsaturated fatty acid composition of all the cellular and subcellular fractions examined. The total concentration of polyunsaturated fatty acids is not altered, the markedly lower level of cervonic acid being compensated for by a higher level of 22:5(n-6). This compensation is quantitative, but total unsaturation remains abnormally low. After switching rats from a diet deficient in α-linolenic acid to a normal diet, the rate of recuperation is remarkably slow; it takes several months for cerebral cells and organelles to recover normal levels of cervonic acid. Cerebral capillaries and microvessels recuperate at the same speed as the rest of the brain, although they are in contact with normal lipoproteins.

A pathogenesis of α-linolenic acid deficiency has been described in the monkey (49) and in humans (14, 50, 52). A deficiency in n-3 fatty acids has been proposed as a syndrome of modern society (53). It is, therefore, very important to verify the precise amount of n-3 acids in the diet. The results of this study indicate that, in order to avoid deficiency, α-linolenic acid should be present at 0.4% of the total dietary energy, in agreement with studies in animals (54) and in humans (14, 55, 56).

Requirements for n-3 acids are very high in humans during the neonatal period (7, 8) and must be supplied to the mother during gestation and then to the newborn. Human milk contains α-linolenic acid and also cervonic acid, which are often absent from infant formula. Human newborns receiving formula have red blood cells that are deficient in cervonic acid (57). The fatty acid composition of red blood cells can serve as an index of cerebral membrane composition (58). In addition, there is undoubtedly a relationship between dietary lipids, serum fatty acids (59) and the properties of red blood cells (60) and their structure (61).

The origin of brain saturated and unsaturated fatty acids (in situ synthesis and dietary origin) is well documented (62); in contrast, polyunsaturated fatty acid metabolism in nerve tissue needs further study.

Polyunsaturated fatty acid deficiencies can appear among newborns fed formula, among the inhabitants of the Third World (in both children and adults), in the elderly fed special diets, in patients fed by enteral and parenteral nutrition, and in surgical patients, whose requirements are often increased.

This study shows that the dietary α-linolenic requirement for membrane synthesis is the same, regardless of the organ (200 mg/100 g diet). In contrast, linoleic acid requirements vary between 150 and 1200 mg/100 g diet, according to the organ (data to be published). Thus, the optimum dietary n-6/n-3 ratio is between 1 and 6, depending upon the organ. We suggest that, for the rat, the minimum α-linolenic and linoleic acid intake should be 200 mg and 1200 mg/100 g diet, i.e., 0.4% and 2.4% of total dietary energy, respectively. If no pharmacological effect is desired, the n-6/n-3 ratio should be ~ 1/6. In view of the relative metabolism of humans and the experimental model animal, their rates of development, their brain/body ratios and the fatty acid composition of their nerve membranes, it is possible to propose that results obtained in the rat could also be valid for humans. However, human requirements are probably higher.

ACKNOWLEDGMENT

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