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# Changes in the Fatty Acid Composition of Brain and Liver Phospholipids from Rats Fed Fat-Free Diet

*Ivančica Delaš*[1]*, *Milivoj Popović*[1], *Tomislav Petrović*[1], *Frane Delaš*[2] *and Davor Ivanković*[1]

[1]School of Medicine, Šalata 3, HR-10 000 Zagreb, Croatia

[2]Faculty of Food Technology and Biotechnology, Pierottijeva 6, HR-10 000 Zagreb, Croatia

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**Summary**

This study has been undertaken with the aim of elucidating the effect of a fat-free diet (FFD), which is known to be deficient in essential fatty acids (EFA), on the composition of fatty acids in the brain and liver glycerophospholipids of rats. Changes in the stereochemical distribution of fatty acids linked to the *sn*-1 or *sn*-2 position were of special interest. Two groups of animals were fed either the control diet (CD) or the FFD for two weeks. From the total lipid extracts of the brain and liver tissues, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol+phosphatidylserine (PI+PS) fractions were separated by column and thin layer chromatography (TLC). After digestion with phospholipase $A_2$ ($PLA_2$), fatty acids from the *sn*-1 and *sn*-2 positions were separately converted into methyl esters and analyzed by gas chromatography. In animals fed FFD, the relative levels of unsaturated fatty acids increased in the *sn*-1 position of the PI+PS fraction in both liver and brain tissues, as well as in the PE fraction from the brain tissue. In other fractions no statistically significant differences were found. When the levels of particular fatty acids were evaluated, significant decreases in the amounts of palmitic (PA, 16:0), stearic (SA, 18:0), and nervonic (NA, 24:1n-9) acids, and/or significant increases of eicosenoic (ENA, 20:1n-9), arachidonic (AA, 20:4n-6) and docosahexaenoic (DHA, 22:6n-3) acids were detected in some fractions. It can be concluded that in the brain and liver glycerophospholipids of rats fed FFD, the EFAs lacking in the diet were moderately substituted by endogenously synthesized unsaturated fatty acids.

*Key words*: fat-free diet, phospholipids, liver, brain, rat, phospholipase $A_2$

## Introduction

During the last 30 years, since the first report on membranes as a fluid mosaic with the phospholipid bilayer as a basic constitutive element (*1*), great interest has been focused on the determination of membrane phospholipid composition, and thus on the physicochemical properties of membranes. The type and fatty acid composition of phospholipids is of great importance for the maintenance of cell integrity and fluidity (*2*). Many authors have shown the effect of decreased rigidity of a phospholipid membrane containing fatty acids with a shorter chain or multiple double bonds (*3*,*4*).

The composition of membrane lipids is complex and depends partially on the cell type, and for a given cell type, it has been considered to be quite constant. Alhough it is obvious that the cell itself regulates the relative ratios of different polar lipids and cholesterol, it is well known that the ratios vary with age and cell cycle, as well as a consequence of environmental influences.

*Corresponding author; Phone: ++385 1 45 66 757; Fax: ++385 1 45 90 236; E-mail: ivancica.delas@mef.hr

Evidence indicates that feeding diets differing in fatty acid composition can induce physiological changes in the membrane function, involving the activity of enzymes, hormone-activated functions and the expression of activity in the cell nucleus (*5,6*). Despite the ability of the human body to synthesize fatty acids necessary for cell structures, some fatty acids, *i.e.* linoleic (LA, 18:2n-6), and linolenic (LNA, 18:3n-3), are essential, as originally reported by Burr and Burr (*7*). Subsequently, many studies revealed that these fatty acids function as constitutive elements, as well as precursors for other long chain polyunsaturated fatty acids and their derivatives. Broadhurst and Cunnane (*8*) and Cunnane (*9*) even set out the idea that food rich in DHA provides brain-specific nutrition and plays a significant role in human brain evolution. Furthermore, Cunnane suggests the reconsideration of the term 'essential fatty acids', originally used for LA and LNA, into the term 'conditionally-indispensable', with the aim to improve the understanding of the function and metabolism of polyunsaturated long-chain fatty acids and their dietary essentiality throughout the whole life (*10,11*).

The most commonly advised dietary intervention for protection against cardiovascular disease is a low or modified fat diet. However, such interventions may have a variety of effects, both positive and negative, on other specific risk factors. Fat-free diets are known to be lacking in EFA and, if followed for a long period of time, can result in the development of essential fatty acid deficiency syndrome (EFADS) with skin lesions and scaliness. Besides insufficient dietary intake, EFAD can also be caused by an increased consumption of fatty acids due to acute liver failure (*12*).

Since obesity has become an epidemic, and the fear of coronary heart disease (CHD), diabetes and other disorders is growing, there is an increasing tendency to exclude fats from the diet. With no intention of undervaluing the hazards of a high fat diet, one should keep in mind the importance of lipid components for the human body, as well as the requirements for essential fatty acids and fat-soluble vitamins.

In our laboratory, Popović and co-workers have been investigating changes in the fatty acid composition of different lipid classes induced by specific diets (*13–15*). Our results generally agree with those of other authors working on similar problems. Having in mind the importance of EFAs and their higher homologues, AA, eicosapentaenoic acid (EPA, 20:5n-3) and DHA, as well as their role in membrane phospholipid functioning, in the present study we extend our investigations to analyze the effects of a two-week FFD on the composition of fatty acids in brain and liver glycerophospholipids, separately for *sn*-1 and *sn*-2 positions.

## Materials and Methods

### *Materials*

The chloroform and methanol used were of HPLC grade and were obtained from Riedel-de Haën AG (Seelze, Germany). The enzyme preparation of $PLA_2$ from *Crotalus adamanteus* venom, lot 99F9520, was purchased from Sigma (Deisenhofen, Germany), and was used without further purification. The phospholipid standards (phosphatidylethanolamine – PE, phosphatidylcholine – PC, phosphatidylinositol – PI, phosphatidylserine – PS, lyso PC, phosphatidic acid) used for TLC were the product of Supelco Inc. (Bellefonte, PA, USA). All other reagents were of analytical grade and were purchased from commercial sources.

### *Animals and diets*

Male Wistar rats, 2 months old, mean body mass 230 g, were used in the study. Animals were divided into two groups (6 animals each) and fed two different diets: (*i*) standard laboratory chow supplied by Pliva, Zagreb, Croatia as the control diet (CD), and (*ii*) the fat-free diet (FFD), prepared as pellets in our laboratory, according to the model of Iritani and Narita (*16*). The vitamin mix used for the FFD was a gift of Vitaminka, Sesvete, Croatia. With the exception of fats, both diets fulfilled nutritional requirements of the animals. The detailed composition of the diets is published in our previous work (*17*). Animals were housed under controlled temperature (20–25 °C) and light conditions (12 h light/12 h dark) with free access to food and water. After two weeks, the rats were euthanized by Ketalar anesthesia by bleeding through the abdominal vein or by heart punction. Organs were rapidly removed, rinsed with cold saline, weighed and stored frozen. The experiment was approved by the ethical committee of the School of Medicine, Zagreb, Croatia.

### *Protein analysis*

Frozen tissues were homogenized by means of an Ultra-Turax at 2000 rpm. In homogenates, soluble proteins were determined according to the method of Lowry *et al.* (*18*).

### *Extraction and separation of lipid classes*

Homogenized tissues were freeze-dried in a Univapo 100 H evaporator coupled to a Unicryo MC 2L (Uniequip, Martinsried, Germany) cold station and total lipids were extracted using the procedure described by Folch *et al.* (*19*). In order to separate phospholipids from neutral lipids, glass columns, 1×30 cm, were packed with 2.5 g of silica gel for chromatography, 0.05–0.2 mm, 70–325 mesh, the product of E. Merck AG, Germany. Total lipid extract was applied on the column and elution was performed by solvent mixtures of increasing polarity as follows: $CHCl_3$ 7.5 mL, $CH_3OH$ 15 mL, $CH_3OH$+2 % $NH_3$ 8.0 mL, $CH_3OH$+5 % $NH_3$ 10.0 mL. Volume fractions (1 mL) were collected and phospholipid-containing fractions (checked by TLC) were combined and concentrated under nitrogen.

### *Phosphorus analysis*

In the extracts, total lipid phosphorus was determined by the Parker and Peterson procedure (*20*).

### *Separation of phospholipid classes*

The separation of phospholipid classes was performed by TLC on glass (10×20 cm) silica gel 60 TLC plates (E. Merck, 5626) with $CHCl_3/CH_3OH/NH_4OH$

(65:25:4, by volume) as the mobile phase (*21*). Phospholipid extracts obtained by column chromatography were quantitatively transferred to TLC plates along a line at 10 mm from the bottom edge of the plate. The front line of the mobile phase was 16 cm from the origin, and separated lipid classes were detected as yellow zones by exposing to iodine vapours. Particular phospholipids were detected relative to authentic standards, containing 20–30 μg/mL of each: PE, PC, PI and PS. Individual phospholipid zones were marked, scraped off and extracted repeatedly with three 4-mL portions of chloroform/methanol (1:1, by volume) mixture. Since the PI and PS fractions co-migrated in most of the samples, we decided to analyze them as a single fraction. Anhydrous $(NH_4)_2SO_4$ was added to the lipid extracts, the samples were left overnight, the sulphate was then removed by filtration, and the extracts were dried in a vacuum.

### *$PLA_2$-catalyzed acidolysis reaction*

Particular phospholipid fractions separated by TLC were treated with phospholipase $A_2$ ($PLA_2$) from *Crotalus adamanteus* venom (*22*). A commercial enzyme preparation with 1000 U was reconstituted in 1 mL of 0.1 M Tris-HCl buffer+0.1 M $CaCl_2$, pH=8.0. Phospholipid samples dissolved in 2 mL of diethyl ether were mixed with 0.5 mL of buffer and 0.020 mL of $PLA_2$ in a screw capped tube and incubated at 37 °C for 3 h. After cooling to room temperature, 2 mL of chloroform/methanol mixture (2:1, by volume) were added, and the sample was then vortexed and centrifuged. The upper phase was discarded while the lower phase was dried with $(NH_4)_2SO_4$ and evaporated under nitrogen.

### *Separation of sn-2 linked fatty acids from lysophospholipid*

After the treatment with $PLA_2$, samples containing lysophospholipid and fatty acids liberated from the *sn*-2 position were quantitatively transferred to TLC plates, accompanied by authentic lipid standards, and developed to a distance of 8 cm from the origin. Chromatography and collection of separated lysophospholipids and free fatty acids were performed in the same way as already described for the separation of phospholipid fractions, with the exception of the mobile phase height.

### *Gas-liquid chromatography (GLC)*

Fatty acid methyl esters (FAME) from lysophospholipids and from free fatty acids obtained by TLC were prepared by using methanolic HCl, *c*(HCl)=1 mol/L, at 100 °C for 5 h (*23*). They were analyzed by a Perkin-Elmer gas-liquid chromatograph, model Sigma 2, equipped with a flame ionization detector and a capillary column AT WAX (Alltech GmbH, Germany), length 30 m, 0.25 mm i.d. and film thickness 0.25 μm. Nitrogen was used as a carrier gas with a flow of 20 mL/min. The oven temperature was increased from 150 to 250 °C at a rate of 5 °C/min, and the final temperature was kept constant for 20 min. The injector and detector temperatures were 250 and 260 °C, respectively. FAME were identified by comparison of their retention times to those of known reference standards (Supelco Inc., Bellefonte, PA, USA) and the results were collected and processed using an Omega 2 working station (PerkinElmer).

### *Statistical analysis*

Statistical analysis of the obtained results was performed using STATISTICA, v. 7.1 program. Because of the small sample size ($N$=6) and non-normal distribution, results of fatty acid analysis were expressed as median±max values and were compared by nonparametric Mann-Whitney $U$ test (*24*). Differences were considered statistically significant at the level of $p<0.05$.

## Results

The detailed nutrient composition of the two diets is presented in Table 1. Since fats were depleted from the experimental diet, the ratio of the other nutrients changed, but the energy intake did not differ significantly between the two groups, with 15.74 and 15.10 kJ/g for CD and FFD, respectively. The control diet was analyzed for fatty acid composition and the results are given in Table 2, confirming the presence of essential fatty acids, LA and LNA. After the 14-day experiment, the body mass gain for the animals on the FFD was 8 %, as compared to 4 % for the animals on the CD. There were no visible changes in the behaviour of the animals or anatomic changes in the investigated organs in either group. Table 3 contains data of the mean mass of the organs, as well as their protein and total lipid phosphorus mass fractions. There were no statistically significant differences found between the two groups.

Liver and brain phospholipids were isolated from the total lipid extracts by column chromatography and separated by TLC into three classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphati-

Table 1. Composition of the control (CD) and the fat-free diet (FFD)

| Main ingredients | CD/% | FFD/% |
|---|---|---|
| Total proteins | 19.37 | 11.94 |
| Total fats | 3.67 | 0.15 |
| Total carbohydrates | 64.82 | 75.33 |
| Total nitrogen | 3.10 | 1.91 |
| Dry mass | 90.96 | 89.33 |

Table 2. Fatty acid composition of the control diet

| Fatty acid | *w*/% |
|---|---|
| 10:0 | 0.11 |
| 12:0 | 0.47 |
| 14:0 | 1.00 |
| 16:0 | 16.50 |
| 16:1n-7 | 0.92 |
| 18:0 | 2.46 |
| 18:1n-9 | 19.20 |
| 18:2n-6 | 50.71 |
| 18:3n-3 | 5.14 |
| 24:0 | 3.49 |

Table 3. Mean organ masses, mass fraction of proteins in wet tissue, and mass fraction of total lipid phosphorus *vs*. tissue proteins of brain and liver from rats fed the control (CD) or fat-free diet (FFD) for two weeks

| Sample | *m*(sample)/g | | *w*(protein)/(mg/g wet tissue) | | *w*(phosphorus)/(μg/mg protein) | |
|---|---|---|---|---|---|---|
| | CD | FFD | CD | FFD | CD | FFD |
| Brain | 1.30±0.14 | 1.23±0.09 | 36.29±7.33 | 28.93±7.55 | 101.2±27.4 | 103.8±11.8 |
| Liver | 10.01±1.14 | 8.35±0.81 | 76.40±9.05 | 96.56±17.59 | 13.6±5.7 | 11.6±2.4 |

Values are expressed as mean±SD of 6 samples

dylinositol+phosphatidylserine (PI+PS). Each phospholipid class was treated with $PLA_2$ and fatty acids released from the *sn*-2 position were separated from lysophosphatides. Fatty acid methyl esters from the *sn*-1 and *sn*-2 positions were analyzed and compared for all separated phospholipid classes, but only those where statistically significant differences were found are shown.

### *Liver fatty acid composition*

The main patterns of fatty acids linked to *sn*-1 or *sn*-2 positions of liver phospholipids were very similar, although the *sn*-2 position was more often occupied by polyunsaturated fatty acids than the *sn*-1 position. In phospholipids isolated from the livers of animals fed FFD, the only statistically notable differences ($p<0.05$) in fatty acid proportions were found on the *sn*-1 position of the PI+PS fraction for arachidonic acid (AA, 20:4n-6) and palmitic acid (PA) (Fig. 1a). The decrease in PA content, accompanied by an increase in AA content, resulted in an increased unsaturated/saturated fatty acid ratio (U:S) at the *sn*-1 position (0.47 and 0.71, respectively). Fatty acids linked to the *sn*-1 position of PE isolated from the treated animals contained a slightly increased ratio ($0.05<p<0.10$) of myristic acid (Fig. 1b), resulting in a minor change in the U:S ratio. While changes in the fatty acid composition of the PC fraction caused by the FFD were not statistically significant (Fig. 1c), the calculated U:S ratio (0.36 and 0.54, respectively) indicates a general increase in the unsaturated fatty acid content. At the same time the fatty acid composition at the *sn*-2 position was unaffected (data not shown).

### *Brain fatty acid composition*

Despite the fact that no statistically significant differences were found in the fatty acid composition of brain PI+PS fraction (Fig. 2a), minor differences were sufficient to affect the U:S ratio, yielding an increase of 25 % in the treated animals. In the PE fraction, changes induced by the FFD were again found at the *sn*-1 position (Fig. 2b). Significant decrease ($p<0.05$) in stearic acid (SA) content was accompanied by significant increases in the ENA and DHA acid ratios ($p<0.05$). These changes resulted in a 31 % increase in the U:S ratio. The fatty acid composition of the PC fraction in the brain of animals fed FFD indicated a decrease of nervonic acid (NA, 24:1n-9), with the significance level of $0.05<p<0.10$ (Fig. 2c). No detectable differences were found in the proportions of fatty acids from the *sn*-2 position (not shown).

## Discussion

Under unfavourable conditions, such as EFAD, the organism tries to substitute the missing components by *de novo* synthesis. In place of lacking LA and LNA, oleic acid is used as a substrate for desaturases and elongases, resulting in eicosatrienoic acid (20:3n-9) synthesis. As a consequence, a decreased ratio of LA and AA in plasma and tissue lipids, with a consequent increase in eicosatrienoic acid ratio, was reported (*16*,*25*–*27*).

Previous works from our laboratory confirmed this tendency, with the effect much more pronounced in neutral lipids than in phospholipids (*13*,*17*). In recent studies, increasing attention has been focused on the significance of phospholipids in cell signalling. A role in signal transduction is attributed to AA and, most probably, to other long chain fatty acids located on the *sn*-2 position of membrane phospholipids (*28*,*29*).

This study was undertaken with the aim of evaluating the impact of FFD on the fatty acid composition of phospholipids in the brain and liver and to look for possible changes in the stereochemical distribution of fatty acids on glycerol backbone. In order to distinguish between fatty acids linked to *sn*-1 and *sn*-2 positions, phospholipids isolated from animals fed either the CD or FFD were digested with $PLA_2$ from *Crotalus adamanteus* venom. Lysophospholipids and liberated fatty acids from the *sn*-2 position were separately subjected to methanolysis, and the FAMEs obtained were analyzed by gas chromatography.

The global fatty acid distribution in phospholipid classes isolated from rats' liver was not affected by a FFD, with palmitic, stearic and oleic acid as the main components. At the *sn*-1 position of the PI+PS fraction, a significant decrease ($p<0.05$) in PA was detected, with concomitant increase ($p<0.05$) in 20:4 species, resulting in an increased U:S ratio. Although the changes were similar, the magnitude of these changes was much smaller in comparison with the previously detected changes induced by the FFD in the fatty acid composition of total liver lipids (*17*). Namely, the amount of saturated PA and/or SA acid decreased, most probably because these fatty acids were being used as precursors for endogenously synthesized unsaturated fatty acids, mostly oleic acid. Knowing that FFD induces $\Delta^6$-desaturase activity, we expected an increase in the eicosatrienoic acid ratio. Instead, in accordance with the results of Tran *et al*. (*30*), certain amounts of DHA were found, while docosapentaenoic acid (DPA, 22:5n-3) was not detected, indicating that most of the acid was either elongated or oxidized, instead of being esterified into phospholipids. In PE and PC molecular species, changes in the fatty acid composition were not statistically significant, with the increase in myristic acid content of PE being the only exception. Similarly, Menguy *et al*. (*31*) showed the relative retention of 18:0–20:5n-3 and 16:0–22:6n-3 PE molecular species, indicating preferential distribution of EFA in PE.

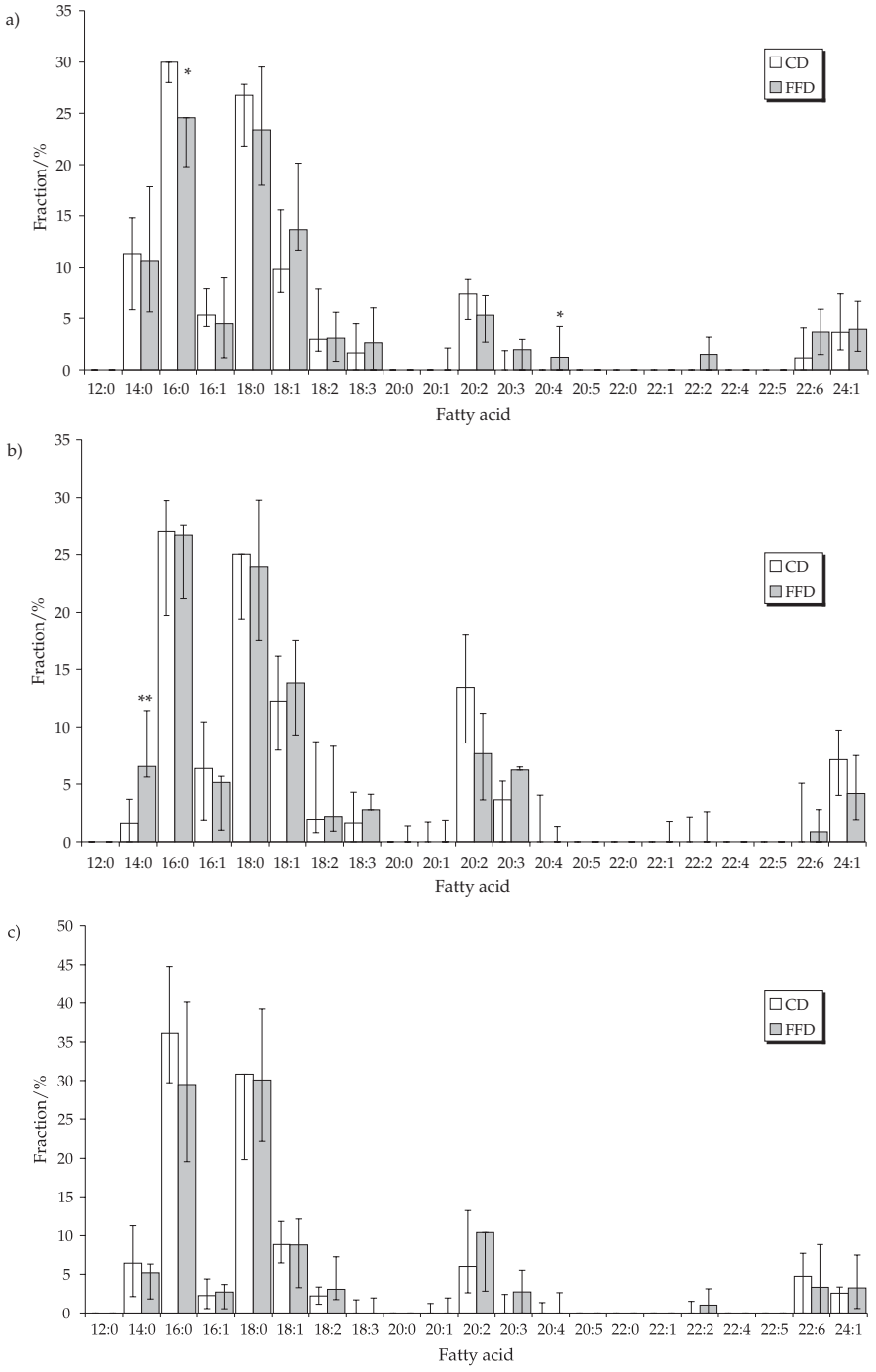


**Fig. 1.** Fatty acid fraction (% of total FA) on the *sn*-1 position of phosphatidylinositol+phosphatidylserine (a), phosphatidylethanolamine (b) and phosphatidylcholine (c) in the liver of rats fed the control (CD) or fat-free diet (FFD) for two weeks. Results are expressed as median±max of 6 samples
Nonparametric Mann-Whitney *U* test was performed to test the difference between medians: * indicates significant difference at $p<0.05$; ** indicates significant difference at $0.05<p<0.10$

As expected, the fatty acid composition of brain phospholipids was even more resistant to disturbances in dietary fats because of the blood-brain barrier. However, a developing brain is much more vulnerable under nutritional insufficiency. It is well known that mother's nutrition during pregnancy strongly influences the brain phospholipid composition and thus the brain function (*32–35*). On the other hand, a number of studies indicate that some neurodegenerative diseases are accompanied by disturbances in fatty acid composition, mostly in glycerophosphatides. Fenton *et al*. (*36*) reviewed clinical research on abnormalities in membrane fatty acid composition and therapeutic trials of fatty acids in schizophrenia. Very interesting observations come from epidemiological studies, which underline the strong correlation between cholesterol lowering diets and increased incidence of suicides, murders and accidents (*37*,*38*). According to Hibbeln and Salem Jr., this could be the result of

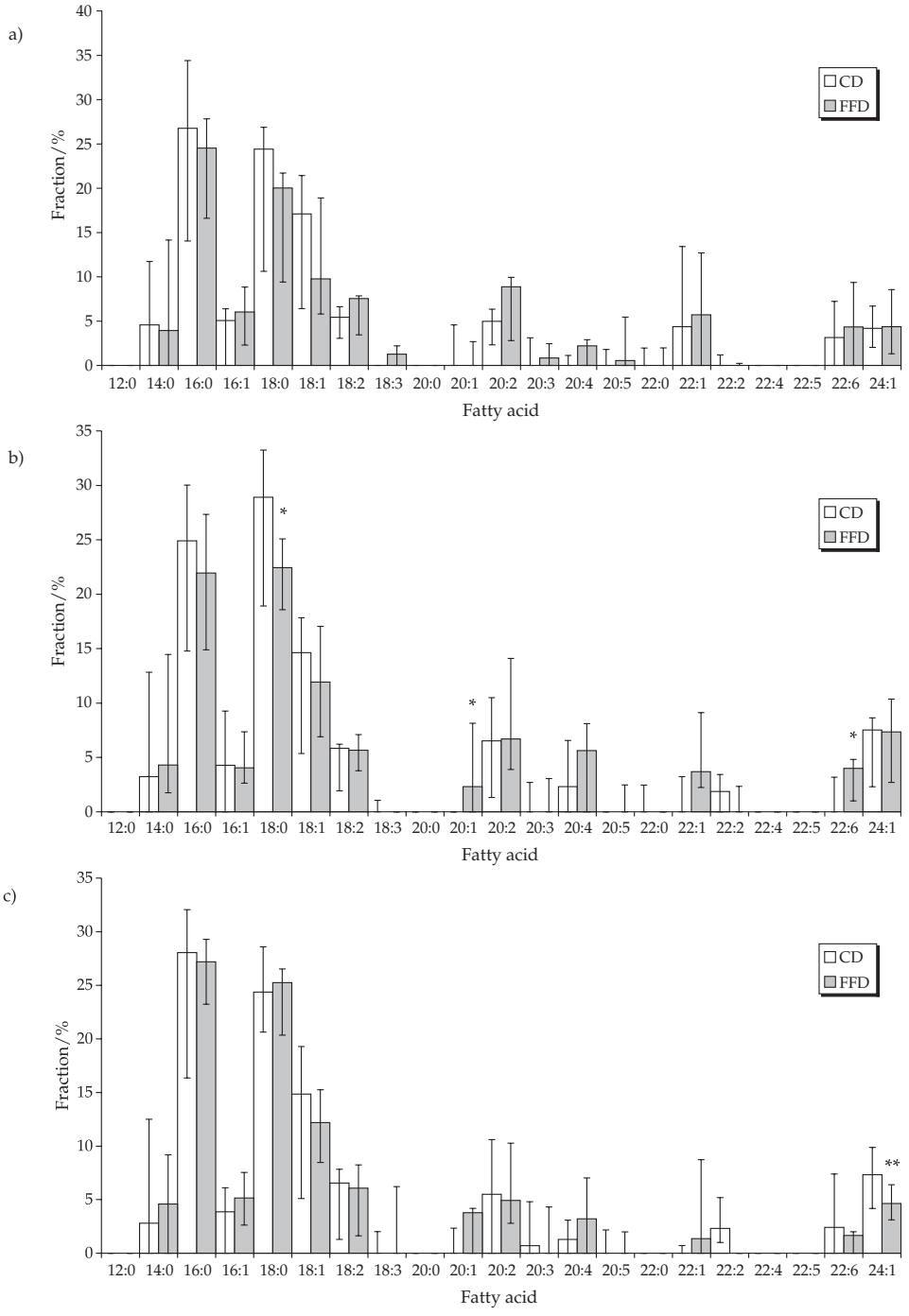


**Fig. 2**. Fatty acid fraction (% of total FA) on the *sn*-1 position of phosphatidylinositol+phosphatidylserine (a), phosphatidylethanolamine (b) and phosphatidylcholine (c) in the brain of rats fed the control (CD) or fat-free diet (FFD) for two weeks. Results are expressed as median±max
Nonparametric Mann-Whitney *U* test was performed to test the difference between medians: * indicates significant difference at $p<0.05$; ** indicates significant difference at $0.05<p<0.10$

n-3 PUFA deficiency as a consequence of low fat/cholesterol lowering diets, applied in prevention of coronary heart disease (*39*). This correlation between dietary n-3 fatty acids deficiency and psychiatry is nowadays generally accepted and well documented (*40,41*).

As already pointed out, in this study short-term FFD caused minor changes in the fatty acid composition of brain phospholipids. The most affected fraction was PE with a significant decrease in SA and concomitant increases in ENA and DHA content. The observed reduction in saturated fatty acid content might be the result of increased catabolism of these acids, and/or it might be the result of their decreased synthesis. Furthermore, considering the increase in eicosenoic acid ratio, it might be the result of increased desaturation and elongation of (decreased) stearic acid. The increase of DHA is somewhat unexpected but may be the result of relative chan-

ges in the overall fatty acid composition and/or of the apparent relative retention of certain long chain PUFA molecular species already seen in liver PE (*31*).

## Conclusions

Taken all together, these results confirm the fact that FFD causes inter- and intramolecular changes in lipid molecules. When such a diet is applied for a short period of time, insufficient dietary intake of EFA can be overcome by endogenous synthesis of polyunsaturated fatty acids, which are no longer of n-3 and n-6, but of n-9 series. This can be explained as a 'tendering' effect, the tendency of the cells to compensate for the disturbances caused by a diet, in order to retain permeability and other physicochemical properties of the original membranes. However, when followed for extended periods of time, *i.e.* as a part of medical treatments for patients with CHD, diabetes or for body mass reduction, low-fat and fat-free diets may be of potential risk. Therefore, if implemented, such diets should be monitored for their effect on essential fatty acids status in patients, and, if necessary, improved by increasing the consumption of foods rich in n-3 polyunsaturated fatty acids and/or by the addition of appropriate supplements.

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