Dietary Cholesterol Induces Changes in Molecular Species of Hepatic Microsomal Phosphatidylcholine

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ABSTRACT: After 21 days on a diet containing 1 g% cholesterol and 0.5 g% cholic acid, rats had an increased content of cholesterol in liver microsomal lipids. In liver, both cholesterol content and Δ9 desaturase activity increased, whereas Δ6 and Δ5 desaturase activities decreased. These changes correlated with increases in oleic, palmitoleic, and linoleic acids and decreases in arachidonic and docosahexenoic acids in total microsomal lipids. Similar fatty acid changes were found in phosphatidylcholine (PC), the principal lipid of the microsomal membrane. In PC the predominant molecular fatty acid species (67% of the total) in the control rats were 18:0/20:4, 16:0/20:4, and 16:0/18:2; and they mainly determined the contribution of PC to the biophysical and biochemical properties of the phospholipid bilayer. The cholesterol diet decreased specifically the 18:0/20:4 species, and to a lesser extent, 16:0/20:4 and 18:0/22:6. The 18:1-containing species, especially 18:1/18:2 and less so 16:0/18:1 and 18:1/20:4, were increased. A new 18:1/18:1 species appeared. The independent effects of the presence of cholesterol and change of the fatty acid composition of the phospholipid bilayer of liver microsomes on the packing were studied by fluorescence methods using 6-lauroyl-2,4-dimethylaminonaphthalene, 1,6-diphenyl-1,3,5-hexatriene and 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene, which test different parameters and depths of the bilayer. Data showed that the increase of cholesterol in the membrane, and not the change of the fatty acid composition of phospholipids, was the main determinant of the increased bulk packing of the bilayer. The increase of fluid oleic- and linoleic-containing species almost compensated for the drop in 20:4- and 22:6-containing molecules. But the most important effect was that the general drop in essential n-6 and n-3 polyunsaturated fatty acids meant that this endogenous source for the needs of the animal decreased.

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Abbreviations: DPH 1,6-diphenyl-1,3,5-hexatriene; ELSD, evaporative light-scattering detector; GLC, gas–liquid chomatography; GP, generalized polarization; HPLC, high-performance liquid chromatography; Laurdan, 6-lauroyl-2,4-dimethylaminonaphthalene; PC, phosphatidylcholine; r_s, steady-state fluorescence anisotropy; r_∞, limiting anisotropy; *S*, order parameter; SRE, sterol-responsive element; SREBP, SRE-binding protein; τ, lifetime; Δτ, differential polarized phase lifetime; τ_M, modulation lifetime; τ_P, phase τ_R, rotational correlation time; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

Cholesterol and phospholipids are obligatory and simultaneous components of many eukaryotic membranes. The cholesterol content of membranes is modified by the amount of cholesterol ingested. We (1,2) and other authors (3) have shown that the ingestion of cholesterol not only increases the cholesterol content of rat liver microsomes but also increases the Δ9 fatty acid desaturase activity that converts stearic to oleic acid and decreases the Δ6 and Δ5 desaturases that convert linoleic and α-linolenic acids to higher polyunsaturated fatty acids. These changes in desaturase activities are correlated with modifications in the percentage of fatty acid in the phospholipids of liver membrane. An increase of oleic acid and a decrease in arachidonic and docosahexaenoic acids were shown. A rise in cholesterol in the microsomes increased the overall rigidity of the microsomal membrane as measured by fluorometric methods (1).

Considering that changes in membrane fluidity produced by different factors can alter the activity or kinetics of intrinsic enzymes and lipid protein interactions (4), as shown for the UDP-glucuronyl transferase (5,6), glucose-6-phosphatase (7,8), fatty acid desaturases (7,8) and NADH-cytochrome-C-reductase (7,8), Leikin and Brenner (1,2) suggested that dietary cholesterol induced rigidity of microsomes could be a factor for the fatty acid Δ9 desaturation increase. In addition to this suggestion, which was verified in experiments in which cholesterol was incorporated *in vitro* in the microsomes (7), it was pointed out that some of this activation effect could be due to a change in desaturase synthesis (1).

This last suggestion was confirmed by Landau *et al.* (9) who showed that cholesterol feeding induced an increase of stearoyl-CoA desaturase RNA in rat liver, but no information was given on the Δ6 and Δ5 desaturase problem and on the mechanism of the changes evoked by cholesterol in microsome fluidity.

We know from preceding publications (1) that the cholesterol diet increases the proportion of oleic acid and decreases the proportions of arachidonic and docosahexaenoic acid in microsomal phospholipids, but we do not know which specific molecules are modified.

In the present work, we addressed this question and also which molecular species of microsomal phosphatidylcholine (PC), the principal phospholipid component of the membrane,

are specifically modified by the change in Δ9, Δ6, and Δ5 desaturase activity as evoked by the high-cholesterol diet.

Moreover, the preceeding publication (1) showed, as expected, that the cholesterol incorporation in the liver microsomal membrane increased the microsomal membrane overall packing. However, at that time, the extent to which the change of fatty acid composition of the bilayer phospholipids, as evoked by the modification of the Δ9, Δ6, and Δ5 desaturation activity, contributed to this effect was not investigated; nor was the cholesterol-specific effect investigated. For these reasons, the biophysical properties of a bilayer prepared only with liver microsomal membrane phospholipids were compared to one prepared with whole lipids. Special probes that tested the properties at different depths of the bilayer were used.

EXPERIMENTAL PROCEDURES

Animals and diets. International regulations for animal care were observed throughout these experiments. Male Wistar rats were separated after weaning into two groups of eight animals each. The control group was fed, as in the experiment of Leikin and Brenner (1), a diet composed of 64 g% starch, 23 g% delipidated casein, and 13 g% corn oil plus 2% vitamins and 4% minerals, as described by Rogers and Harper (10). The test group was fed the same diet as the control, with the addition of 1 g% cholesterol and 0.5 g% cholic acid. The reason for the cholic acid addition to the food was to increase cholesterol absorption in the intestine. Ingestion of a small amount of cholic acid did not alter the microsomal membrane composition (Brenner, R.R., unpublished). The animals were pair-fed, and both groups gained the same amount of weight. After an experimental period of 21 d, the animals were killed by decapitation without anesthesia and exanginated. The liver from each animal was rapidly excised and placed in an ice-cold homogenizing solution (1:3 wt/vol) composed of 0.25 M sucrose, 1 mM EDTA, and 10 mM phosphate buffer (pH 7.2). Microsomes were obtained by differential ultracentrifugation at 100,000 × *g* (Beckman Ultracentrifuge) as described elsewhere (11). They were kept stored at –80°C.

Protein concentration was measured according to the procedure of Lowry *et al.* (12).

Lipid analysis. Lipids were extracted from microsomes according to the procedure of Folch *et al.* (13). Total lipid content was measured by aliquot evaporation to constant weight. Cholesterol content was determined by the procedure of Huang *et al.* (14), and total phosphorus by the method of Chen *et al.* (15).

Total phospholipids were separated from nonpolar lipids by absorption on activated silicic acid. Between 8 and 10 mg of total lipids, dissolved in 3 mL of chloroform, was mixed with 25 times their weight of silicic acid. Nonpolar lipids were washed out twice with chloroform.

PC and other phospholipid classes were separated from total lipids by high-performance liquid chromatography (HPLC) using an evaporative light-scattering detector (ELSD) (16). An Econosil silica column of 10 μm and 250 × 4.6 mm from All-tech Associates (Deerfield, IL), was used. Elution was performed at a flow rate of 1 mL/min by a gradient of hexane/isopropanol/dichloromethane (40:48:12, by vol) to hexane/isopropanol/dichloromethane/water (40:42:8:8, by vol) for 15 min followed by additional elution with the latter solvent for 30 min. Solvents were previously sonicated to eliminate air. Lipids (300 μg) dissolved in 50 μL of mobile phase were injected.

Nebulization in the ELSD was set at 90°C drift tube temperature and 2.20 L/min of nitrogen gas flow to the nebulizer the PC peak was collected manually from the column effluent using a flow splitter. The solvent was evaporated under N_2 and redissolved in methanol/triethylamine (2:1).

PC molecular species separation. The separation of the molecular species was done by using the method of Brouwers *et al.* (17). In this case, triethylamine proved to be very efficient for the removal of specific interactions between the phospholipid headgroup and HPLC column material. Resolution of molecular species was performed on two 5 μm endcapped Lichrosphere 100-RP18 columns in series obtained from Merck (Darmstadt, Germany). Isocratic elution was applied with a solvent composed of methanol/acetonitrile-triethylamine (58:40:2, by vol) at a flux of 1 mL/min. The detection and quantification were done in an ELSD using N_2 as nebulizer gas at a flux of 1.8 L/min and a temperature of 100°C (17).

A sample of 1 mg of PC was injected; 5 parts out of 100 went to the detector, and the remaining materials of the peaks were collected and identified by gas–liquid chromatography (GLC) analysis.

Fatty acid compositions of both PC and molecular species were analyzed by GLC in a Hewlett-Packard 5840 apparatus after esterification with methanol. A 10% SP2330 column (Supelco Inc., Bellefonte, PA) packed on Chromosorb WAW was used. The temperature was programmed to obtain a linear increase of 3°C/min from 140 to 220°C. The chromatographic peaks were identified by comparison of the retention times with those of standards.

Fluorescence measurements. (i) Fluorescent probes. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma Chemical Co. (St. Louis, MO). 6-Lauroyl-2,4-dimethylaminonaphthalene (Laurdan) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Eugene, OR).

(ii) Liposome preparation. Two types of liposomes were prepared, one with the total microsomal lipids and the other with the phospholipids. Lipids (0.8 mg) in $CHCl_3$ were added to a rounded-bottom glass tube. $CHCl_3$ was evaporated under a N_2 stream, and 1 mL of buffer A (Tris-HCl 0.1 M, NaCl 0.15 M, pH 8.0) was added. After 15 min at room temperature to allow hydration, samples were vigorously vortexed for 2 min. Then the lipid suspensions were extruded 11 times through a 100-nm-pore filter using a LiposoFast extruder (Avestin, Inc., Ottawa, Ontario, Canada).

(iii) Liposome labeling. Extruded liposomes (0.25 mL) were mixed with 0.25 mL of a $4 \cdot 10^{-6}$ M suspension of the fluorescent probe (DPH, TMA-DPH, or Laurdan) in buffer A and vigorously vortexed. Samples were kept at room temper-

ature for at least 30 min, and then diluted 5 times with buffer A before the fluorescent measurements were made. The final concentration of the samples was 80 μg/mL of lipids and $4 \cdot 10^{-7}$ M of the fluorescent probe.

(iv) Fluorescence measurements. All measurements were made in an SLM 4800 spectrofluorometer in 1 × 1 cm cuvettes.

DPH and TMA-DPH fluorescence. DPH or TMA-DPH steady-state fluorescence anisotropy (r_S), lifetime (τ) and differential polarized phase lifetime (Δτ) were measured using an excitation wavelength of 361 nm and observing the total emission at wavelengths >389 nm through a sharp cut-off filter (KV389) according to Lakowicz *et al.* (18,19) with some modification (20–22). For the τ and Δτ measurements, the exciting light was modulated sinusoidally in amplitude at 18 or 30 MHz with a Debye-Sears modulator and vertically polarized with a Glan-Thompson polarizer. For τ, the emission was observed through a Glan-Thompson polarizer oriented 55° to the vertical to eliminate the effect of Brownian rotation (23). The phase shift and demodulation of the emitted light were measured relative to the reference standard 1,4-bis(5-phenyloxazol-2-yl)benzene in ethanol (τ = 1.35 ns) (24) and used to compute the phase (τ_P) and modulation (τ_M) lifetimes of the samples (25). Δτ was obtained from the phase shift between the parallel and perpendicular components of the emission observed with the emission polarizer vertically or horizontally oriented, respectively. Data were interpreted according with the model of hindered wobbling rotation (26). The values obtained for r_S, τ, and Δτ were used to compute: (i) the rotational correlation time (τ_R), which is inversely related to the rotational rate and reflects the local viscous resistance to the probe rotation; and (ii) the limiting anisotropy (r_∞), which is related to the order parameter S ($S2 = r_\infty/r_o$) and reflects the limitation imposed by the local environment to the extent or range of the probe wobbling. τ_R was obtained from the positive solution of Equation 1:

$$0 = A\,\tau_R^2 + B\,\tau_R + C \qquad [1]$$

where,

$$A = \Delta\tau\,[\,\omega^2\,\tau^2\,(1 + 2\,r_o)\,(1 - r_o) + (1 + 2\,r_S)\,(1 - r_S)\,] - 3\tau\,(r_o - r_S) \qquad [2]$$

$$B = \tau\,[\,2\,\Delta\tau\,(1 + 2\,r_S)\,(1 - r_S) - 3\tau\,(r_o - r_S)\,] \qquad [3]$$

$$C = \tau^2\,\Delta\tau\,(1 + 2\,r_S)\,(1 - r_S) \qquad [4]$$

where r_o is the fundamental anisotropy (0.39 for DPH and TMA-DPH) (27) and $\omega = 2\,\pi \times F$, F being the modulation frequency in hertz. r_∞ was calculated from

$$r_\infty = r_S - (r_o - r_S)\,\tau_R/\tau \qquad [5]$$

Laurdan fluorescence. Intensity and generalized polarization spectra were taken essentially with monochromator bandpasses of 8 nm in excitation and emission as previously described (28). All spectra were corrected for background contribution by subtracting the signal of unlabeled samples. An excitation wavelength of 360 nm was used for emission intensity spectra and an emission wavelength of 430 nm was used for excitation intensity spectra. Generalized polarization (GP) spectra were obtained by measuring the excitation intensity spectra using 440 nm (I_{440}) and 490 nm (I_{490}) for the emission, and the emission intensity spectra at 340 nm (I_{340}) and 410 nm (I_{410}) excitation wavelength. GP in the excitation (exGP) and emission (emGP) bands were obtained from $exGP = (I_{440} - I_{490})/(I_{440} + I_{490})$ and $emGP = (I_{410} - I_{340})/(I_{410} + I_{340})$, respectively.

RESULTS

Effect of cholesterol on microsomal lipid composition of liver. Increased ingestion of cholesterol for 21 d produced an increased content of cholesterol in the liver microsomal lipids (Table 1), as shown by Leikin and Brenner (1). However, it did not decrease either the total phospholipid percentage (Table 1) or the relative proportion of different phospholipid classes (data not shown). The ratio of microsomal lipid to protein was not significantly different (0.56 ± 0.08 for the control animal vs. 0.58 ± 0.10 for the cholesterol-treated rats).

The relative fatty acid composition by weight of total lipids in liver microsomes from cholesterol-fed rats was very different from control animals (Table 2); these results were similar to those of Leikin and Brenner (1). A decrease in palmitic and stearic acids was shown, together with a sharp

TABLE 1
Composition of Cholesterol and Total Phospholipids in Microsomal Lipids of Rat Liver After Cholesterol Feeding[a]

	Control (wt%)	Cholesterol-fed (wt%)
Cholesterol	4.84 ± 0.57	8.25 ± 1.44[b]
Total phospholipids	58.00 ± 4.15	60.78 ± 5.40

[a]Results are the mean ± SD of six animals analyzed separately.
[b]Data on cholesterol are compared to control using the Student's *t* test $P < 0.001$.

TABLE 2
Effect of Cholesterol on Fatty Acid Composition (wt%) of Fatty Acids of Total Microsomal Lipids

Fatty acids	Control	Cholesterol	*P*
16:0	14.96 ± 2.14	12.42 ± 0.83	<0.01
16:1n-7	0.48 ± 0.40	1.10 ± 0.55	<0.05
18:0	20.19 ± 3.07	13.80 ± 1.66	<0.001
18:1n-9	9.94 ± 1.74	19.27 ± 2.27	<0.001
18:2n-6	14.46 ± 1.62	16.49 ± 1.22	<0.01
20:3n-9	1.12 ± 1.02	1.56 ± 0.51	
20:3n-6	1.07 ± 0.74	2.32 ± 0.47	<0.005
20:4n-6	24.46 ± 2.40	21.26 ± 1.86	<0.01
22:4n-6	3.27 ± 2.74	3.49 ± 0.68	
22:4n-3	4.22 ± 1.22	4.29 ± 0.57	
22:5n-3	1.27 ± 0.96	1.42 ± 0.25	
22:6n-3	4.56 ± 2.43	2.59 ± 0.63	<0.05

[a]Only principal fatty acids were considered. Data are the mean of eight animals ± SD of the mean.

increase in oleic acid that correlates with the enhancement of the Δ9 desaturase activity already shown in other works (1,9). An increase in linoleic acid and a decrease in arachidonic acid, essential n-6 fatty acids, and a decrase in docosahexaenoic acid of n-3 family would be in accordance with the experimentally determined decrease of Δ6 and Δ5 desaturase activities, which diminish their biosynthesis, as shown by Leikin and Brenner (1,2).

The effect of cholesterol diet on fatty acid composition of microsomal PC (Fig. 1) follows a similar pattern to total lipid fatty acids (Table 2). A decrease in palmitic and stearic acids, which are substrates of the Δ9 desaturase, and an increase in palmitoleic and oleic acids, their enzymatic products, were shown. Similarly to Table 2, an increase in linoleic acid and a decrease in arachidonic acid (which is the product of Δ6 and Δ5 desaturation of linoleic acid) were found along with a decrease of docosahexaenoic n-3 acid.

Effect of cholesterol on microsomal PC molecular species. The application of the method of Brouwers *et al.* (17) to separate molecular species of microsomal PC gave a very good resolution and quantification for them. Eleven major peaks were separated and identified for the control animals and 12 for the cholesterol-fed rats (Fig. 2). The distribution by weight percentage of the molecular species of PC is given in Table 3. As expected, no fully saturated species were detected. The majority of the species were constituted by saturated and unsaturated acids and even some of them by two unsaturated acids. The major species found were 18:0/20:4, 16:0/20:4, and 16:0/18:2, which are in accordance with the fatty acid composition of PC (Fig. 1). The same predominance was found in the PC of other liver and kidney organelles such as the nucleus (Ves Losada, A., Maté, S.M., and Brenner, R.R., unpublished data). Therefore, we must admit that these three molecular species, and mainly 18:0/20:4, are the ones that particularly determine the contribution of PC molecules to the biophysical properties and structure of the phospholipid bilayer in liver microsomes of animals fed a corn-oil diet. Undoubtedly, they also determine possibly important membrane points susceptible to oxidation.

The effect of cholesterol feeding, as expected produced on the PC composition (Fig. 1), an important change not only in the proportion of the different molecular species but also an indication of an interchange of the components.

The decrease of arachidonic acid biosynthesis from linoleic acid (1,2) evoked by cholesterol feeding would apparently explain the notable decrease of the 18:0/20:4 molecular species and the lesser decrease in 16:0/20:4, also favored by the decrease of 16:0 and 18:0, substrates of the increased Δ9 desaturase.

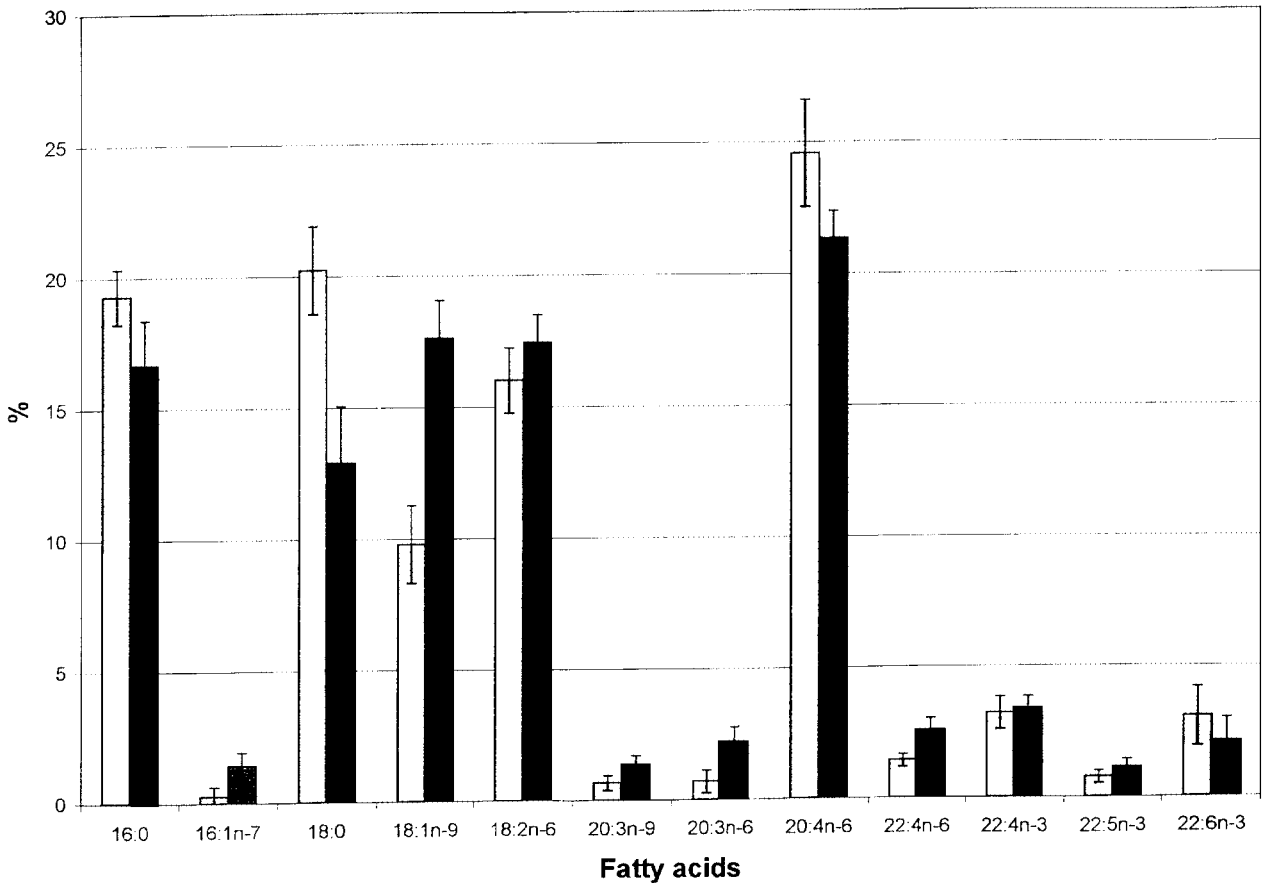

FIG. 1. Fatty acid composition (wt%) of rat liver microsomal phosphatidylcholine (PC). Results are the average of six animals analyzed individually ± SD. Open bars correspond to control animals; black bars, to cholesterol-fed rats.

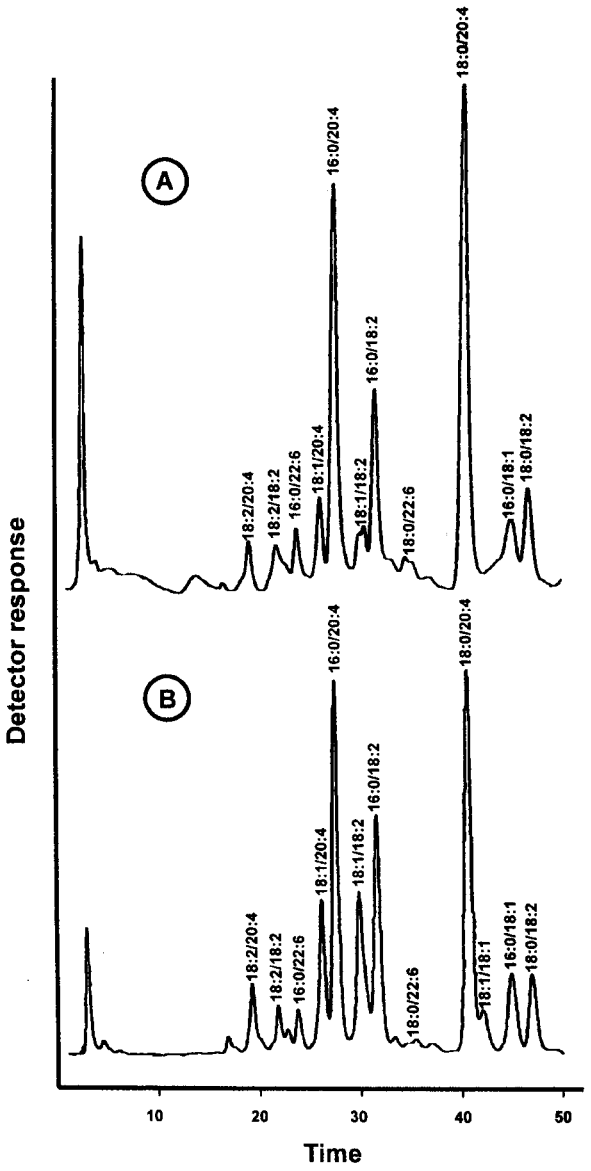

FIG. 2. High performance liquid chromatogram of PC molecular species carried out as explained in the Experimental Procedures section in two Lichrosphere 100-RP 18 columns. Peaks separated and identified by gas–liquid chromatography. (A) Control; (B) cholesterol-treated. For abbreviation see Figure 1.

However, the small increase in the minor species 18:2/20:4 and 18:1/20:4 could be due to the enhancement of 18:2 and 18:1 components as a consequence of the decrease of Δ6- and increase of Δ9-desaturase activities (1,2). It is also notable that 16:0/18:2 species did not change, probably owing to the compensatory effects of the 16:0 decrease and 18:2 increase in cholesterol-treated rats. The decrease in docosahexaenoic n-3 acid biosynthesis could be the cause of a major decrease in the 18:0/22:6 molecular species and the smaller one in 16:0/22:6.

The increase in Δ9 desaturase (1,9) evoked by the cholesterol administration enhanced not only the18:1-containing species 18:1/20:4, 18:1/18:2, and 16:0/18:1 but also the appearance of a new 18:1/18:1 molecular species. These changes produced compensation and stability of phospholipid liposome fluidity, matter that will be discussed later.

TABLE 3
Distribution by Weight Percentage of Phosphatidylcholine Molecular Species of Control and Cholesterol-Fed Rats

Molecular species	Control	Cholesterol	*P*
18:2/20:4	2.03 ± 0.81	4.31 ± 0.90	<0.001
18:2/18:2	1.58 ± 0.73	2.66 ± 0.94	<0.05
16:0/22:6	3.03 ± 1.24	2.51 ± 0.82	
18:1/20:4	3.87 ± 1.60	8.74 ± 1.41	<0.001
16:0/20:4	18.68 ± 2.36	16.41 ± 1.32	<0.05
18:1/18:2	5.13 ± 2.13	12.24 ± 1.70	<0.001
16:0/18:2	15.73 ± 2.93	15.75 ± 2.86	
18:0/22:6	3.00 ± 1.83	0.78 ± 0.72	<0.01
18:0/20:4	32.56 ± 3.53	20.11 ± 5.24	<0.001
18:1/18:1	0.00 ± 0.00	2.40 ± 1.99	<0.01
16:0/18:1	5.53 ± 0.60	7.29 ± 0.90	<0.001
18:0/18:2	8.86 ± 2.14	6.80 ± 1.14	<0.05

[a]Molecular species were separated and quantified by high-performance liquid chormatography as described in the Experimental Procedures section. Results represent the average of seven animals ± SD of the mean.

Although 18:0/20:4, 16:0/20:4, and 16:0/18:2 are still the predominant PC molecules in cholesterol-treated rats, 18:1/18:2 and to a lesser extent 18:1/20:4 and 16:0/18:1 species also play an important role in the contribution of PC to the properties and structure of the lipid bilayer.

Packing changes produced by diet in the cholesterol-containing and pure phospholipid liposomes of microsomal membranes studied by fluorometric methods. The influence of dietary cholesterol on the lipid bilayer dynamical properties was studied by measuring the dipolar relaxation of Laurdan and the rotational diffusion of DPH and TMA-DPH, which test different zones of the bilayer. With the aim of distinguishing between the specific effect of cholesterol content and that of the change in the phospholipid acyl chain composition, these measurements were made in large unilamellar vesicles composed of total microsomal lipids and in those composed of only microsomal phospholipids.

The effects of Laurdan on the fluorescence excitation and emission spectra of both types of vesicles are shown in Figure 3. No large differences were found in the excitation spectra. In contrast, the emission spectrum, which is very sensitive to the acyl chain packing, is significantly changed by the treatment in total microsomal vesicles. In these vesicles, dietary cholesterol evoked an increase in the blue-shifted unrelaxed emission at 440 nm. The blue-shifted emission, however, disappears almost completely in the vesicles made with only microsomal phospholipids, and no large effect is observed after the treatment. These Laurdan spectral changes can be quantified by the GP parameter calculated as explained in the Experimental Procedures section. GP spectra are shown in Figure 4. On the excitation band, the GP calculated between emission wavelengths of 440 and 490 nm is increased by the dietary cholesterol in the total lipid vesicles. In the cholesterol-free phospholipid vesicles, the GP values are much

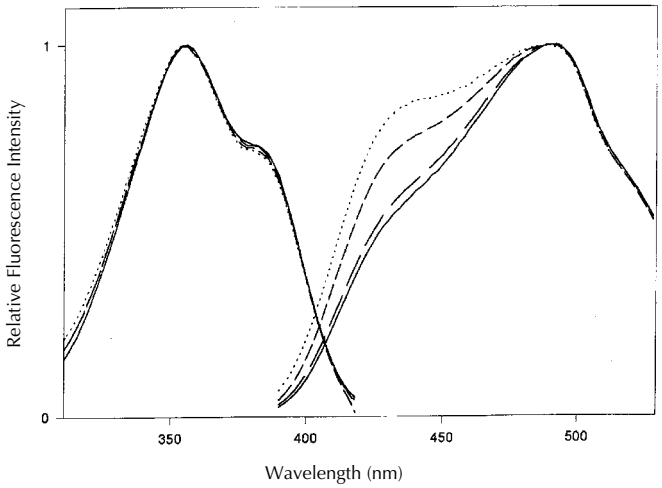

FIG. 3. Normalized excitation and emission fluorescence spectra of 6-lauroyl-2,4-dimethylaminonaphthalene (Laurdan) in unilamellar vesicles of microsomal phospholipids of control (——) and treated (— —) rats, and unilamellar vesicles of total microsomal lipids of control (– – –) and treated (·····) rats.

lower, and only a small increase in GP is observed for the phospholipid vesicles of treated microsomes with respect to the controls.

Thus, the effect of dietary cholesterol on lipid packing is mainly due to changes in the cholesterol content, and only a small effect is due to the change produced in the fatty acid composition.

Additional information on the lipid bilayer dynamical properties was obtained by studying the rotational diffusion of the neutral probe DPH, which locates deep in the bilayer, as well as of the charged TMA-DPH, which is anchored to the polar interface. These results are shown in Table 4. The steady-state anisotropy (r_s) of DPH is increased in the vesicles of total microsomal lipids of rats fed the cholesterol-rich diet. This is in accordance with previous results of Leikin and Brenner (1). Complete information on the probe rotational behavior cannot be obtained from the r_s parameter alone, since it depends on the fluorescence lifetime and is affected by both the rate and extension of the rotation. Additional lifetime and differential polarized phase measurements allow to one calculate the correlation time (τ_r), which is inversely related to the rotational rate, and the limiting anisotropy (r_∞), which is related to the wobbling extension and therefore to the membrane order. The influence of dietary cholesterol on the behavior of DPH in total lipid vesicles is mainly due to a change in the wobbling extension as indicated by marked changes in the r_∞ parameter. The rate of DPH rotation is also slowed by dietary cholesterol as shown by the change of the correlation time. DPH r_s is lower in the cholesterol-free phospholipid vesicles and in this case, on the contrary, no effect of diet is observed. The r_∞ and τ_r were also maintained in the phospholipid liposomes in spite of the change of diet.

Contrary to DPH, the r_s of the TMA-DPH probe is not changed by dietary cholesterol in other total lipid liposomes on phospholipid liposomes. The differential polarized phase measurements, however, indicated that a significant increase in TMA-DPH r_∞ is produced by the cholesterol diet only in total lipids.

Not only fluorescence lifetime is necessary to allow the calculation of the rotational parameters, but also it can inform on the probe environment polarity and water penetration into the

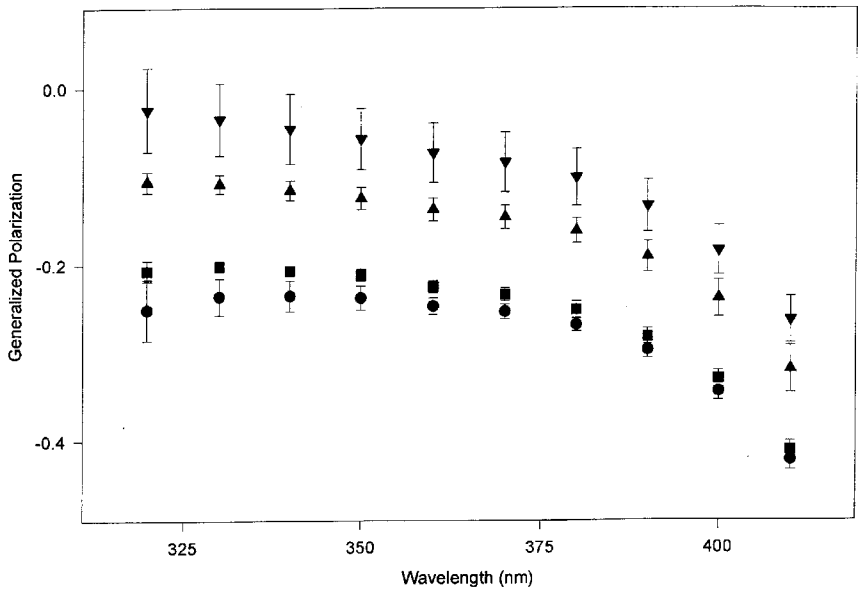

FIG. 4. Generalized polarization along the excitation band of Laurdan fluorescence in unilamellar vesicles of microsomal phospholipids of control (●) and treated (■) rats, and unilamellar vesicles of total microsomal lipids of control (▲) and treated (▼) rats. See Figure 3 for abbreviation.

TABLE 4
Effect of Dietary Cholesterol on the Lifetime and Rotational Parameters of DPH and TMA-DPH in Unilamellar Vesicles of Phospholipids and Total Lipids of Rat Liver Microsomes

		DPH				TMA-DPH			
Samples		r_S[a]	τ_P (ns)	τ_R (ns)	τ_∞	r_S[a]	τ_P (ns)	τ_R (ns)	τ_∞
Phospholipids	Controls (1)	0.066 ± 0.002	7.23 ± 0.08	1.00 ± 0.03	0.021 ± 0.002	0.192 ± 0.005	2.93 ± 0.10	1.39 ± 0.10	0.098 ± 0.012
	Treated (2)	0.069 ± 0.003	6.78 ± 0.08	1.03 ± 0.05	0.020 ± 0.002	0.197 ± 0.005	2.80 ± 0.05	1.46 ± 0.07	0.096 ± 0.008
Total lipids	Controls (3)	0.079 ± 0.005	7.27 ± 0.24	1.04 ± 0.05	0.034 ± 0.006	0.194 ± 0.008	3.49 ± 0.07	1.44 ± 0.19	0.113 ± 0.021
	Treated (4)	0.090 ± 0.003	7.02 ± 0.24	1.15 ± 0.02	0.041 ± 0.003	0.200 ± 0.008	3.40 ± 0.12	1.27 ± 0.12	0.134 ± 0.013
Statistics[b]	1 vs. 2	n.s.	$P < 0.01$	n.s.	n.s	n.s.	n.s.	n.s.	n.s.
	3 vs. 4	$P < 0.05$	n.s.	$P < 0.01$	n.s	n.s	n.s	n.s	$P < 0.05$
	1 vs. 3	$P < 0.01$	n.s.	n.s.	$P < 0.05$	n.s.	$P < 0.001$	n.s.	n.s.
	2 vs. 4	$P < 0.001$	n.s.	$P < 0.01$	n.s.	$P < 0.01$	$P < 0.05$	$P < 0.05$	$P < 0.01$

[a]Data are the mean ± standard deviation of four samples. Data were obtained from measurements at 18 MHz. Similar results were obtained at 30 MHz modulation frequency.
[b]Significance levels obtained from unpaired (1 vs. 2 and 3 vs. 4) and paired (1 vs. 3 and 2 vs. 4) Student's *t*-test. DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)1-6-phenyl-1,3,5-hexatriene.

lipid bilayer. DPH lifetime is only slightly decreased by dietary cholesterol. However, after cholesterol elimination, DPH lifetime is notoriously shorter in the microsomal phospholipid vesicles of animals treated with the cholesterol-rich diet. This indicates that changes in fatty acid composition produced by treatment resulted in a more polar environment for DPH in the phospholipid vesicles. This fact may be a consequence of packing defects that allow an increased water penetration in the bilayer interior. However, when cholesterol is present, it would eliminate them by decreasing water penetration. In the case of the externally located TMA-DPH probe, the fluorescence lifetime is significantly shorter in the phospholipid vesicles than in the total lipid ones, showing the sealing effect of cholesterol against water penetration. However, in contrast to DPH, no dietary effect and therefore no molecular species change were observed on the TMA-DPH lifetime.

DISCUSSION

The ingestion by the rate of 1% dietary cholesterol evokes in the rat its incorporation into the animal's tissue including liver microsomes (Table 1), a well-documented increase in microsomal Δ9 desaturation of fatty acids, and a decrease of Δ6- and Δ5-desaturase activity (1–3,9). Whereas Leikin and Brenner (1,2) suggested the increase of Δ9 desaturation of fatty acids was the consequence of the increased packing produced by the cholesterol incorporation to the membrane, Landau *et al.* (9) presented data showing that the stearoyl-CoA desaturase RNA increases, to some extent, before the microsomal cholesterol incorporation into microsomes. This effect was investigated during short periods of time (up to 7 d), and results indicated that the enzyme synthesis was modified at the beginning of the event, in part as a result of an increase of the transcription rate as discussed by the authors (9). This result did not discard the possibility that a later viscotropic regulation of desaturase activity might be exerted by the incorporation of cholesterol to the microsomal membrane. Moreover, this fact has been shown to be produced on the Δ9 desaturase by *in vitro* incorporation of cholesterol into microsomes (7) and into other microsomal enzymes (5–8). Besides, the effect of a downward shift of temperature on membrane fluidity may be the triggering cause for the activity change in desaturase genes (29).

Ntambi (30) recently indicated that the transcriptional activation of genes containing sterol-responsive element (SRE) is under the regulation of sterols through modulation of the proteolytic maturation of the SRE-binding proteins (SREBP-1 and SREBP-2). In sterol-rich cells, proteolytic cleavage of SREBP inserted in the endoplasmic reticulum to the N-terminal mature polypeptide form does not take place. Therefore, its entrance to the nucleus, its binding to the SRE, and the activation of the Δ9 desaturase are avoided. This conclusion is deduced from cell studies (31). These interesting and important *in vitro* results do not agree with the *in vivo* results already cited (1–3,9) showing that dietary cholesterol activates the Δ9 desaturation. Therefore, more investigation on this subject is necessary to elucidate the cause for this apparent discrepancy and the possible direct or indirect effect of microsomal cholesterol on the membrane-bound Δ9 desaturase.

In fact, whichever mechanism is evoked upon Δ9-, Δ6-, and Δ5-desaturase activities by cholesterol ingestion, the fatty acid composition of the total lipids of liver microsomes is changed (Table 2) in such a way that it correlates with an increase of Δ9-desaturase activity and with a decrease in Δ6- and Δ5-desaturase activities, as shown experimentally (1–3,9). The amount of Δ9-desaturase substrates, 16:0 and 18:0, is decreased, whereas the amount of products, 18:1 and 16:1, is increased. Correspondingly, the amount of Δ6 desaturase substrate, linoleic acid, is increased whereas the amount of products of Δ6 and Δ5 desaturases, arachidonic and docosahexaenoic acids, is decreased. As expected, similar results are shown in the fatty acid composition of the microsomal PC (Fig. 1).

These effects on fatty acid composition, produced by a cholesterol-rich diet, of liver total microsomal lipids and PC

are apparently general since they have already been found by Veno and Okuyama in total liver and plasma (32). It is difficult to suppose that these changes might be due to the movement of lipids between organelles. Moreover, since the fatty acid composition of control and cholesterol-fed rats is the same from our point of view, the more plausible cause for the fatty acid changes is the modification of the desaturase activity already demonstrated (1–3,9). Of course, not all lipid classes and not all organs will show identical change. The possible effect of fatty acid capture by esterification to cholesterol as well as of specificity of enzymes involved in different phospholipid synthesis and retailoring and even fatty acid oxidation may also alter the final composition of the phospholipid species and PC molecular species.

PC is the principal phospholipid of liver endoplasmic reticulum. PC coerces other lipids into a bilayer structure and is a major determinant of the biophysical properties of phospholipid bilayers. Moreover, since the proportion of phospholipid classes was not changed in the present experiment due to the cholesterol diet, we may admit that the changes of PC composition would reflect, in some way, the biophysical changes of the phospholipidic part of the microsomal membrane.

After 21 d of the cholesterol diet, the composition of the molecular species of liver microsomal PC had reached an equilibrium, and the liver microsomal membrane had been adapted to the new situation. The following phenomena were produced in the membrane: (i) the incorporation of cholesterol increased the packing of the microsomal membrane, shown by an increase in the GP of Laurdan-labeled microsomal lipids (Fig. 4) and in the fluorescence r_∞ of DPH and TMA-DPH labeled ones (Table 4); (ii) there was a decrease in the Δ6 and Δ5 fatty acid desaturation and an increase of Δ9 desaturation, which is already well-documented (1–3,9); and (iii) there was a change of the relative ratios of palmitic, stearic, oleic, linoleic, arachidonic, and docosahexaenoic acids in phospholipid molecules like PC. These changes modified the proportion of PC molecular species. However, these changes were produced in such a way that the fluidity of the phospholipidic part of the membrane was roughly maintained, as shown by the fluorescence properties of Laurdan (Figs. 3 and 4), DPH, and TMA-DPH (Table 4).

The decrease in arachidonic acid (Fig. 1) led to a decrease mainly in the 18:0/20:4 PC molecular species, but also to a lesser extent in 16:0/20:4 that is more fluid. The 16:0/20:4 species is more fluid since it shows less rotational correlation and fluorescence anisotropy of DPH-labeled species as shown by Tricerri *et al.* (20) and Garda *et al.* (33). It also correlates with a decrease in saturated fatty acids, especially stearic acid. However, the even more fluid 18:2/20:4 species increased, along with the 18:2/18:2 species, which corresponds to an enhancement of 18:2n-6 acid (Fig. 1). Linoleic acid was fully provided by the diet, and the cholesterol diet decreased its conversion to arachidonic acid.

In this way, the effect of the decrease of highly polyunsaturated fatty acid-containing species on phospholipid membrane rigidity has been reduced to a minimum. It is even better compensated by the increase of oleic acid-containing species 18:1/20:4, 18:1/18:2, 16:0/18:1, and the appearance of a new species of 18:1/18:1 (Table 3).

As it is known, whereas saturated acids produce less fluid phospholipids with increasing chain length (20,33), polyunsaturated acids evoke only slightly more fluid liposomes than monounsaturated ones (34). Therefore, the substitution of 20:4-containing species by 18:1-containing species nearly compensates for the fluidizing effect. These results are similar to ones found in the molecular species of all phosphoglycerides of hepatic microsomal lipids of rats fed on a fat-free diet (33). Although the effect in this case was due not to cholesterol but to a dietary deficiency of fats that evoked an essential fatty acid-deficient status, the change in the availability of fatty acids to build up the membrane phospholipids was rather similar. There was an increase of oleic acid and palmitic acid and a decrease of arachidonic, stearic and obviously and differentially, linoleic acid. The eicosatrienoic n-9 acid was also enhanced by an increased biosynthesis. The distribution of the resulting molecular species was similar to the cholesterol effect, and it showed an important decrease of the 18:0/20:4n-6 species and a lesser one for 16:0/20:4n-6. Both were the major molecular species found in PC. In compensation, 16:0/18:1, 16:0/20:3n-9, and 18:0/20:3n-9 species were increased. The final result was that this molecular compensation in the phosphoglycerides in fact avoided a change in the packing of the microsomal phospholipid bilayer measured by fluorescent methods and it was similar to our actual case.

The change in the fatty acid composition found in the PC molecular species (Table 3) indicates that the type and proportion of the fatty acids at both the *sn*-1 and *sn*-2 positions had been altered. This is a consequence not only of the availability of different fatty acids but also of the changes in the specific activity of enzymes involved in PC biosynthesis. A re-tailoring process was produced; and a deacylation-reacylation at carbon *sn*-2 through a phospholipase A_2 and an acyltransferase known as Lands pathway (35) is apparent. Remodeling of PC molecular species at the *sn*-1 position of glycerol (36) also takes place. Moreover, the contribution of a *de novo* synthesis of phospholipids is also important considering especially the change in the proportion and type of fatty acids at the *sn*-1 position. The profile of PC species might also be influenced by different types of pathways of synthesis such as the phosphatidylethanolamine methylation.

In conclusion, we suggest that the re-tailoring of PC, as well as other phosphoglyceride molecular species, evoked by a high-cholesterol diet was able to nearly maintain the liquid-crystalline structure and bulk fluidity of the phospholipidic part of the microsomal membrane. However, it was unable to compensate for the striking packing effect produced by the incorporation of cholesterol molecules in the membrane.

Although the bulk average biophysical properties of the phospholipidic part of the microsomal membrane did not change, we found different molecular phospholipidic composition that un-

doubtedly alters other microscopic properties of the membrane, e.g., its chemical reactivity and possibly the interaction with proteins. The substitution, in part, of arachidonic and docosahexaenoic acid PC-containing species for oleic and linoleic-containing species evoked by cholesterol diet decreases the density of double bonds in the proximity of the membrane border, modifies the position and number of the clouds of polarizable π electrons surrounding the double bonds, and alters possible effects on membrane bound proteins. However, the most important effect produced by the fall in the amount of molecular species containing arachidonic and docosahexaenoic acids is the decrease of the endogenous source of these acids for their specific functions in brain and retina, as well as the activation of nuclear receptors, synthesis of eicosanoid, hepoxiline and lipoxine, etc.

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