Role of Cholesterol in the Microsomal Membrane[1]

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It is generally assumed that cholesterol is the public enemy of health. However, in many respects cholesterol is a crucial molecule that serves essential functions in humans. Cholesterol is the substrate for the biosynthesis of bile acids and of steroidal hormones, including gluco- and mineral-corticoids, sex hormones and progesterone. In addition, 7-dehydrocholesterol is a precursor of vitamin D_3. Cholesterol is also an important component of biological membranes. Membranes are generally thought to consist of phospholipid bilayers into which membrane proteins are embedded. Yet, cholesterol molecules are present in most animal membrane structures. Due to its amphipathic nature bearing an OH-group and a hydrocarbon skeleton with rigid rings and a branched chain of eight carbons, cholesterol is perfectly suited to mesh with lipid bilayers.

The cholesterol molecule has evolved from the lanosterol structure by demethylation of the α face thus creating a surface that is more suitable to serve its function in membranes (Fig. 1). In some membranes, the cholesterol/phospholipid ratio is very high. It is 0.83 in liver plasma membranes (1) and 0.90 in erythrocyte membranes (2). In rat liver microsomes (3) it is 0.30, but it can be elevated to 0.61 by administering cholesterol to rats for 21 days at a level of 1% of the diet. Therefore, the liver microsomal membrane is a convenient model to study the effect of cholesterol at different concentrations on the composition, physical properties and enzyme kinetics of membranes.

An increase in the cholesterol concentration in a lipid bilayer evokes a condensing effect at temperatures higher than the transition temperature of the bilayer. This causes a decrease in the rotational diffusion of the hydrophobic fatty acid chains of phospholipids and a decrease in the translational diffusion.

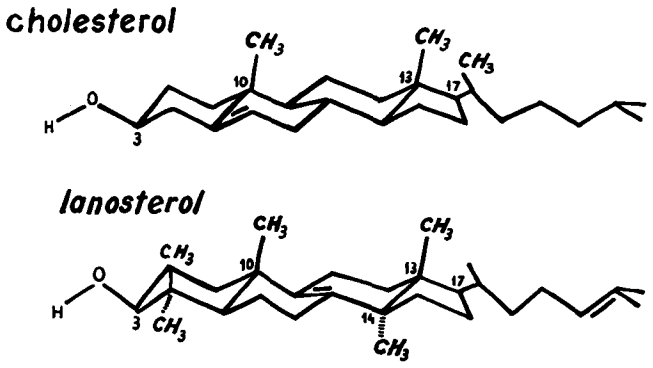

FIG. 1. Structure of cholesterol and lanosterol.

We measured rotational diffusions in liver microsomal membranes by determining the stationary fluorescence anisotropy (r_s) of microsomes labeled with diphenylhexatriene (DPH) and we observed an increase in r_s that depended on the amount of cholesterol that had been incorporated (4) (Fig. 2). The relationship is

$$r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities parallel and perpendicular to the plane of polarization of the excitation beam. The result indicated that with increasing cholesterol levels, a decrease in the rate and range of rotational diffusion occurred.

Lateral diffusion was investigated by labeling the microsomes with pyrene and by measuring the quotient of the fluorescence intensities of the excimer *vs* the monomer formed. This quotient is dependent on the proportion of pyrene and on the collisions of the molecules that depend on lateral diffusion. We observed a decrease in the slopes due to cholesterol incorporation which is indicative of a decrease in lateral diffusion (Fig. 2).

EFFECT OF CHOLESTEROL ON URIDINE DIPHOSPHATE (UDP)-GLUCURONYLTRANSFERASE

With above results in hand, we studied the effect of increased cholesterol levels in the diet on the lipid

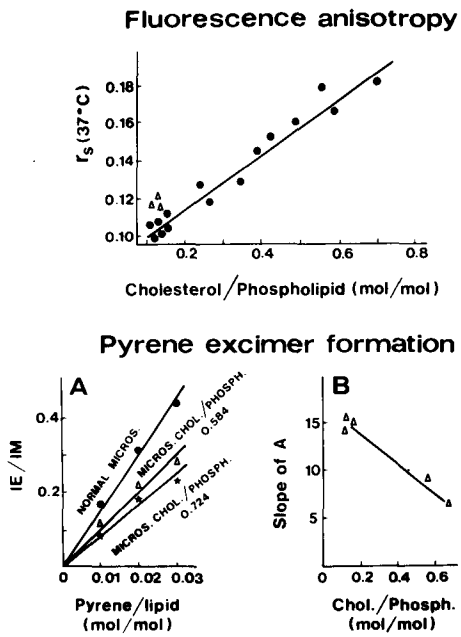

FIG. 2. Effect of cholesterol on rotational and translational diffusion in microsomes. Rotational diffusion was measured by the fluorescence anisotropy of diphenylhexatriene-labeled microsomes. Membranes (approx. 40 μg of lipid/mL) were labeled with $2.5.10^{-7}$M diphenylhexatriene. In the pyrene excimer formation, Ie/Im was obtained from the fluorescence intensities at 472 nm and 392 nm of microsomes labeled with pyrene at different cholesterol concentrations.

[1]Supelco Award Address presented at the 81st AOCS Annual Meeting held in Baltimore, MD, April 1990.

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Abbreviations: DPH, diphenylhexatriene; ESR, electron spin resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; UDP, uridine diphosphate.

composition of liver microsomes in guinea pigs. We chose the UDP-glucuronyltransferase as enzyme because it is membrane-bound and its activity is known to be modified by the lipid environment.

Administration of cholesterol at 3% of the diet for twenty days increased the cholesterol/phospholipid and phosphatidylcholine/phosphatidylethanolamine molar ratios of guinea pig liver microsomes, but it did not modify, in general, the other lipids. Also, the fatty acid composition essentially was maintained (5) (Table 1). The changes that were observed were accompanied by a decrease in the fluidity of the bulk lipids of the membrane.

A significant effect resulting from these membrane changes was exerted on UDP-glucuronyltransferase activity. This enzyme is bound to the liver microsomal membrane and catalyzes the transfer of the glucuronyl group from the UDP.GA to *p*-nitrophenol. The reaction proceeds in a random-ordered sequence in which two substrates are involved. Therefore, 4 K_m values were measured (Fig. 3). It was found (Table 2) that cholesterol incorporation into the membrane decreased the 4 K_m values and increased the maximal velocity of the reaction, indicating a change in the affinity of the enzyme for the substrates and an increase in enzyme activity. However, the changes produced were more fundamental as the Hill coefficient of the reaction was increased from 0.40 to 0.68. The Hill coefficient is commonly used to measure the cooperativity of a reaction, with a coefficient <1 indicating a non-Michaelian reaction and negative cooperativity. Cholesterol incorporation into the membrane promoted a reaction change from non-Michaelian to Michaelian that correlated with a decrease in bulk membrane fluidity.

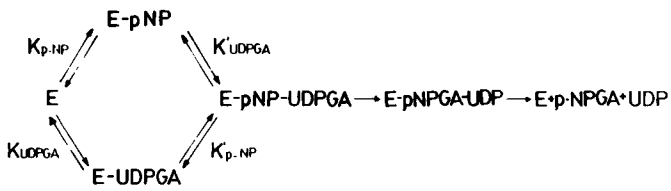

FIG. 3. Postulated reaction mechanism for UDP-glucuronyl transferase.

TABLE 1

Effect of Dietary Cholesterol (3%) on Lipid Composition of Guinea Pig Liver Microsomes (% weight)[a]

	Control	+ Cholesterol
Cholesterol	13.4 ± 0.4	28.7 ± 2.3
Cholesteryl esters	2.8 ± 0.1	4.6 ± 0.8
Triacylglycerol	3.6 ± 0.2	3.9 ± 0.9
Phosphatidylcholine	47.8 ± 1.8	41.5 ± 1.9
Phosphatidylethanolamine	25.3 ± 0.4	13.7 ± 0.5
Phosphatidylinositol	7.1 ± 0.1	7.6 ± 0.6
Double bonds/sat. acid	2.97 ± 0.03	3.04 ± 0.04
Cholesterol/Pcholine mol/mol	0.31 ± 0.10	0.46 ± 0.08
Pcholine/Pethanolamine mol/mol	1.75	2.78

[a]Lipids were separated and determined by thin-layer chromatography. Results are the mean of five animals analyzed separately ± S.E.

TABLE 2

Effect of Dietary Cholesterol on the r_s of Microsomal Membrane and Kinetic Constants of UDP-Glucuronyltransferase[a]

	Normal	+ Cholesterol
r_s	0.112 ± 0.004	0.131 ± 0.003
K_{UDPGA}(mM)	12.3 ± 0.4	8.1 ± 0.3
K'_{UDPGA}(mM)	10.6 ± 0.2	7.2 ± 0.1
$K_{p.NP}$(mM)	0.12 ± 0.03	0.07 ± 0.01
$K'_{p.NP}$(mM)	0.10 ± 0.01	0.06 ± 0.01
Vmax (nmol/min/mg prot)	10.5 ± 1.1	15.9 ± 1.3
Hill coefficient	0.40 ± 0.02	0.68 ± 0.03

[a]Fluorescence anisotropies (r_s) of diphenylhexatriene were measured at 37°C. Results are the mean of five animals analyzed separately ± S.E. K_{pNP} and K_{UDPG} correspond to the dissociation constants for the binary complex and K'_{pNP} and K'_{UDPGA} correspond to the dissociation constants for the ternary complex as shown in Figure 3.

TABLE 3

Effect of *in vitro* Change of Guinea Pig Microsomal Cholesterol Content on the Fluidity of the Membrane and Kinetic Properties of UDP-Glucuronyltransferase[a]

	−Cholesterol	Normal	+Cholesterol
Chol/Phosph	0.270	0.368	0.513
r_s	0.102	0.112	0.154
K_{UDPGA}(mM)	13.1	12.0	8.9
K'_{UDPGA}(mM)	10.9	10.3	6.2
Kp.NP (mM)	0.20	0.17	0.10
K'p.NP (mM)	0.15	0.12	0.08
Vmax (nmol/min/mg prot)	8.7	10.1	14.3
Hill coefficient	0.38	0.47	0.74

[a]Results are the mean of five animals analyzed separately ± S.E.

In vivo incorporation of cholesterol caused not only an increase in cholesterol/phospholipid ratio and viscosity of the microsomal membranes, but also a change in the phosphatidylcholine/phosphatidylethanolamine ratio. To determine whether or not this latter change could evoke the observed effect on the UDP-glucuronyltransferase, a similar change in cholesterol content of the microsomes was brought about by *in vitro* incorporation. *In vitro* incorporation or deprivation of cholesterol in microsomes was accomplished by use of a cytosolic cholesterol transfer protein in the presence of cholesterol-enriched or cholesterol-deprived liposomes. Similar results were obtained upon cholesterol incorporation or depletion of the microsomal membrane which decreased or increased, respectively, the fluidity of the bulk bilayer lipids which, in turn, affected the kinetics of the UDP-glucuronyltransferase reaction (Table 3) (6).

Zakim and colleagues (7) using a delipidated and purified UDP-glucuronyltransferase reconstituted the enzyme with lecithins in the liquid-crystalline state or in the gel phase and observed that the reaction was non-Michaelian with lipids in the gel phase, but Michaelian with lipids in the liquid-crystalline phase. These results,

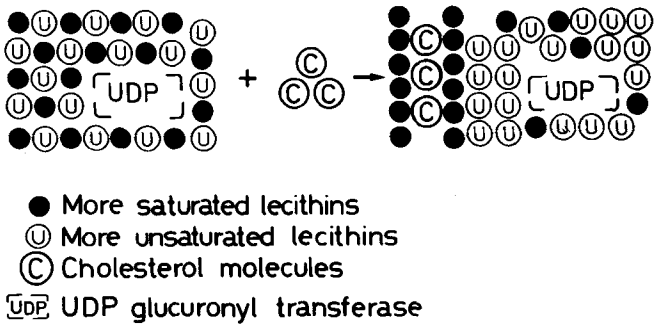

FIG. 4. Possible selective effect of cholesterol on phospholipid distribution in lipid bilayers.

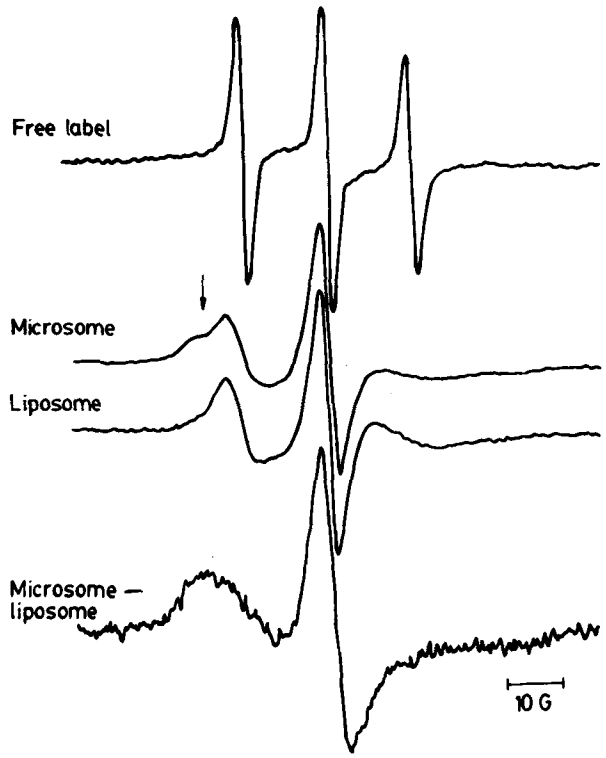

FIG. 5. ESR spectra of 3-doxyl-androstan-17-ol free and incorporated in guinea pig microsomes and liposomes. Arrow points to the shoulder produced by immobilized lipids.

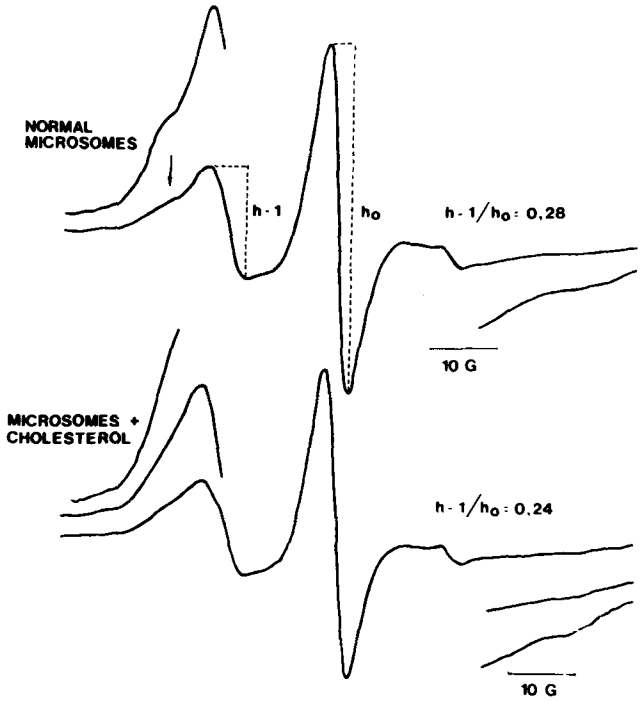

FIG. 6. Effect of cholesterol on ESR spectra of 3-doxyl-androstan-17-ol in guinea pig microsomes. Arrow points to the shoulder produced by immobilized lipids.

when compared with ours, indicate that one must differentiate between the bulk lipids of the membrane and the lipids bound in the enzyme domain.

It is entirely possible that in our experiments cholesterol incorporation into the membrane, while increasing the r_S and the order of the bulk lipids, would produce a fluidizing effect on the bound lipids. This effect could be produced by cholesterol if it would exert selectivity for specific phospholipids with lesser fluidity. It could, for example, associate preferentially with saturated lecithins, leaving behind unsaturated molecules that would remain in the enzyme domain (Fig. 4). In this respect, Melchior and Scautto (8), as well as Jain (9) and Presti *et al.* (10) have shown that cholesterol incorporation into a lipid membrane causes separation of cholesterol-phospholipid domains from pure phospholipid domains. Moreover, Gruyer and Bloch (11) have found that cholesterol increases the microviscosity of saturated phosphatidylcholine vesicles than the viscosity of unsaturated vesicles.

Additional information can be obtained by electron spin resonance (ESR). We labeled both liver microsomes and liposomes prepared from microsomal lipids with 3-doxyl-androstan-17-ol and studied the ESR spectra. The spectrum of the free label shows three peaks and indicates substantial mobility. Doxyl-androstan-17-ol incorporation into the microsomes decreased mobility as judged by the main spectrum, but gave rise to a small shoulder that corresponded to the lipids surrounding the microsomal proteins. This shoulder did not appear when the probe was incorporated in the pure microsomal lipids. By subtraction of the spectra, it was possible to obtain the spectrum of the protein-bound lipids (Fig. 5). Addition of cholesterol to the labeled microsomes resulted in a general decrease in the mobility of the main spectrum representative of the bulk lipids and caused a decrease in h_{-1}/h_0 ratio. However, it also caused the disappearance of the shoulder of the immobilized lipids (Fig. 6).

The interaction of cholesterol in microsomes and its effect on the UDP-glucuronyltransferase reaction may be representative of what generally happens in biological membranes. We suggest that cholesterol incorporation into a membrane, due to the specific affinity of cholesterol for selected lipids, can alter the composition of the bulk lipid phase and modify the microenvironment and properties of specific membrane proteins.

EFFECT ON FATTY ACID DESATURASES

The fatty acid desaturases are integral enzymes of the microsomal membranes which have been shown to be

responsive to cholesterol. There are three important desaturases: the Δ6, Δ5 and Δ9 desaturases that catalyze the formation of double bonds in Δ6, Δ5 and Δ9 position of fatty acids. All require the presence of the microsomal electron transport system that consists of the integral proteins NADH cytochrome b_5 reductase and cytochrome b_5 that transports electrons from NADH to the desaturase. All three components of the system are embedded in the microsomal membrane and, therefore, may be affected by the fluidity of the lipid bilayer.

In these experiments, we used rat liver because the fatty acid composition of guinea pig liver was not altered by the cholesterol diet. In rat, 1% dietary cholesterol administered for 21 days resulted in a significant change in the proportion of unsaturated acids in liver microsomes (3). After 21 days of cholesterol administration, the cholesterol/phospholipid molar ratio of the rat liver microsomes was increased from 0.30 to 0.61, and the bulk lipid fluidity, (as expressed by the fluorescence anisotropy of DPH) was markedly decreased (the r_s increased) (Fig. 7). The proportion of oleic acid was increased, whereas the proportions of arachidonic, eicosapentaenoic (20:5n-3) and docosahexaenoic acids (22:6n-3) were markedly decreased (Fig. 8). The effect was reversible (12) as depletion of cholesterol reversed the trends.

The changes in unsaturated fatty acid composition undoubtedly indicate a change in Δ9, Δ6 and Δ5 desaturase activities (Fig. 7). In fact, when the desaturase activity was measured, we found that cholesterol incorporation into the membrane increased Δ9 desaturase activity and increased Δ6 and Δ5 desaturases. The changes were reversible. Thus, *in vivo* cholesterol incorporation into rat liver microsomes decreases fluidity but increases phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratios resulting in an increase in the desaturation of stearic acid to oleic acid and a decreased conversion of linoleic acid to arachidonic acid and of α-linolenic acid to eicosapentaenoic and docosahexaenoic acids.

Using liposomes enriched with cholesterol in the presence of a cytosolic cholesterol transfer protein, we produced *in vitro* incorporation of cholesterol into the microsomal membrane. Under these conditions (Fig. 9) we observed a decrease in membrane fluidity and an increase not only in the Δ9 desaturase activity, but also in Δ5 and Δ6 desaturase activities (4). The PC/PE ratio was not changed. Therefore, the same viscotropic effect was apparently produced *in vitro* by cholesterol incorporation on all three desaturases. We also observed that when we fluidized the microsomes by *in vitro* n-butyl or isoamyl alcohol treatment, a decrease in Δ9 and in Δ6 desaturase activity was brought about (13).

In the *in vivo* experiment, cholesterol incorporation increased not only the viscosity of the membrane but also the PC/PE molar ratio. Therefore, it is probable that this phospholipid change causes a decrease in the activities of Δ6 and Δ5 desaturases, which would overlap with the viscotropic effect. In order to test this, we fed rats for 21 days with a diet containing 5% of a β-sitosterol-campesterol mixture (14). This diet resulted in an increase in phytosterol in the membrane and a decrease in the fluidity of the microsomes. However, the PC/PE ratio was not changed. When the activities of the desaturases were measured, we found that the three desaturases were activated, as was observed in the *in vitro* experiments

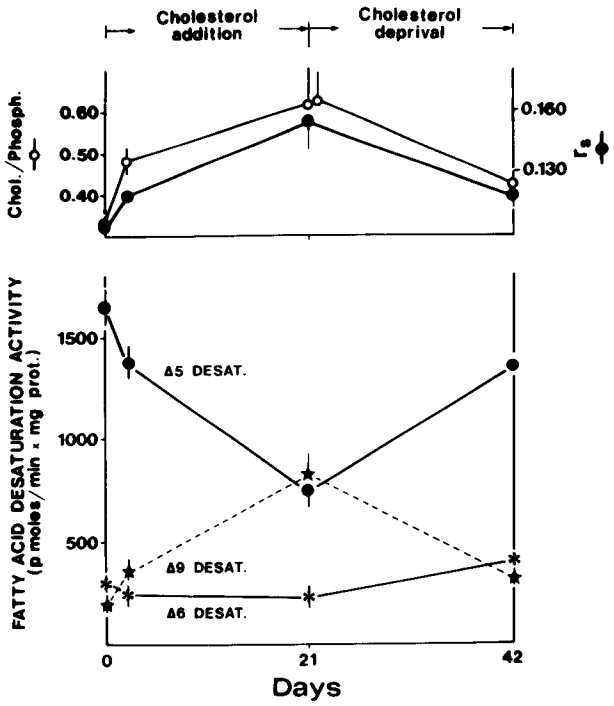

FIG. 7. Effect of dietary cholesterol on the cholesterol/phospholipid ratio, r_s and Δ9, Δ6 and Δ5 desaturase activities of rat liver microsomes. Δ6 Desaturation was measured by the microsomal [1-^{14}C]linoleic acid conversion to γ-linolenic acid; Δ5 desaturation was measured by the conversion of [1-^{14}C]20:3n-6 to arachidonic acid and Δ9 desaturation was measured by the conversion of [1-^{14}C]-palmitic acid to palmitoleic acid.

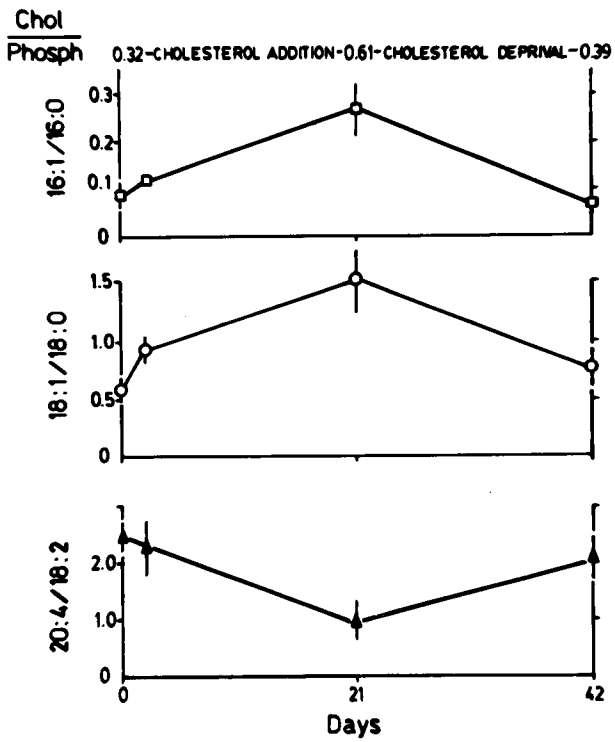

FIG. 8. Effect of dietary cholesterol on the fatty acid composition of rat liver microsomes.

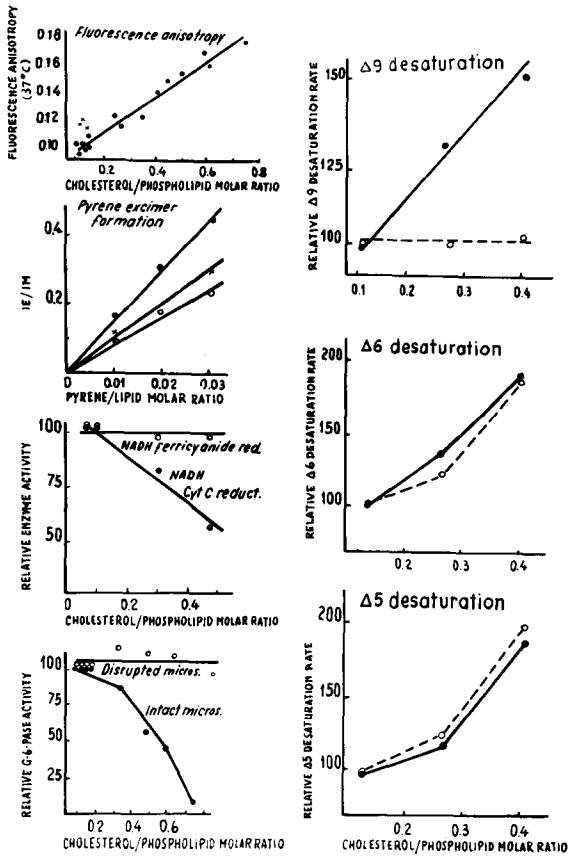

FIG. 9. Effect of cholesterol enrichment of microsomes *in vitro* on fluorescence anisotropy and pyrene excimer formation, electron transport system of microsomes, Δ9, Δ6 and Δ5 desaturation of fatty acids and glucose-6-Pase. Pyrene excimer formation: Cholesterol/phospholipid was equal to 0.120 mol/mol (●—●); 0.584 mol/mol (X—X); and 0.724 mol/mol (O—O). The fatty acid desaturases were tested at 2 μM (●—●) and 66 μM (O—O) concentration of substrate (4).

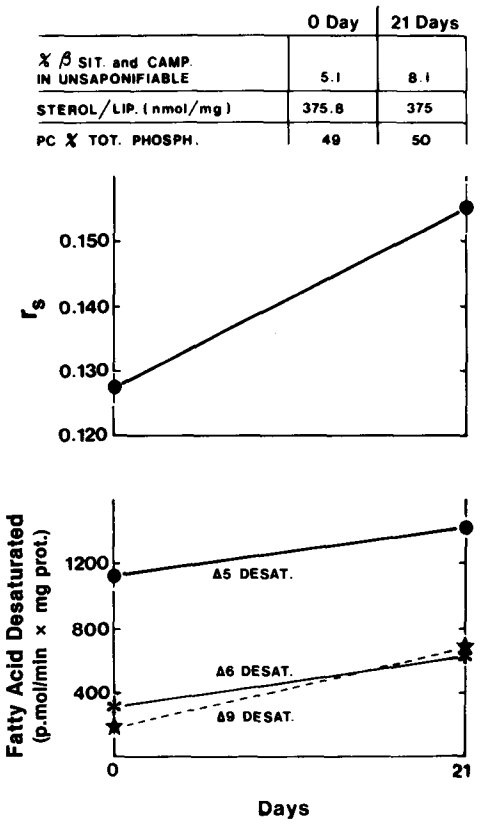

FIG. 10. Effect of 5% dietary campesterol (40%), β-sitosterol (60%) during 21 days on lipid composition, fluorescence anisotropy and Δ9, Δ6 and Δ5 desaturase activity of rat liver microsomes.

(Fig. 10). Therefore, the viscotropic effect was shown only in those instances in which the PC/PE ratio was not increased. When the cholesterol diet was used, just the opposite was observed. Therefore, the activities of Δ5 and Δ6 desaturases would not be regulated by a viscotropic change *in vivo*, as are the Δ9 desaturase, except in the case when the PC/PE ratio was not changed.

Activation of the Δ9, Δ6 and Δ5 desaturases by the phytosterol diet modified the fatty acid composition of liver microsomes as shown by an increase of the ratios of 16:1/16:0 and 18:1/18:0, as well as by an increase in 20:4n-6, 20:5n-3, 22:6n-3 and in the ratio of 20:4n-6 plus 22:4n-6/18:2.

The effects of cholesterol on both the UDP-glucuronyltransferase and fatty acid desaturases of liver microsomes illustrates that diet can cause chemical as well as physical membrane changes that result in specific modifications of membrane-bound enzymes. Some of these changes are due to viscotropic alterations, but others involve changes in the microenvironment of the membrane-bound enzyme.

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