Biochemistry 1991, 30, 9492–9497

Cholesterol Modulation of Lipid–Protein Interactions in Liver Microsomal Membrane: A Spin Label Study†

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Received November 29, 1990; Revised Manuscript Received July 8, 1991

ABSTRACT: ESR spectra of spin probes were used to monitor lipid–protein interactions in native and cholesterol-enriched microsomal membranes. In both systems composite spectra were obtained, one characteristic of bulk bilayer organization and another due to a motionally restricted population, which was ascribed to lipids in a protein microenvironment. Computer spectral subtractions revealed that cholesterol modulates the order/mobility of both populations in opposite ways, i.e., while the lipid bilayer region gives rise to more anisotropic spectra upon cholesterol enrichment, the spectra of the motionally restricted population become indicative of increased mobility and/or decreased order. These events were evidenced by measurement of both effective order parameters and correlation times. The percentages of the motionally restricted component were invariant in native and cholesterol-enriched microsomes. Variable temperature studies also indicated a lack of variation of the percentages of both spectral components, suggesting that the motionally restricted one was not due to protein aggregation. The results correlate well with the effect of cholesterol enrichment on membrane-bound enzyme kinetics and on the behavior of fluorescent probes [Castuma & Brenner (1986) *Biochemistry 25*, 4733–4738]. Several hypothesis are put foward to explain the molecular mechanism of the cholesterol-induced spectral changes.

The great complexity of biological membranes can be understood in terms of functional requirements, since each lipid species seems to be present for a distinct purpose. Among these, cholesterol contributes to the maintenance of membrane stability and the reduction of nonspecific leakage through the bilayer, by filling the free volumes in the hydrocarbon core (Houslay & Stanley, 1982). The molecular understanding of this behavior is based on the investigation of cholesterol effects on both lipid physical properties and lipid–protein interactions. Though the first mechanism is now well-characterized through studies with artifical and natural membranes (Schroeder, 1984; Straume & Litman, 1987), the cholesterol modulation of membrane enzymes and even the cholesterol–protein interaction is still controversial (Yeagle et al., 1988). Evidence has been presented for band 3 (Klappauf & Schubert, 1977) and human erythrocyte glycophorin (Yeagle, 1984) suggesting a direct interaction with the sterol molecule, and exchange experiments indicate a cholesterol–protein interaction in brush border membrane from intestine (Bloj & Zilversmit, 1982). On the contrary, for β-hydroxybutyrate dehydrogenase (McIntyre & Fleischer, 1984), Ca^{2+} ATPase (Silvius et al., 1984), and the nicotinic acetylcholine receptor (Jones & McNamee, 1988), the sterol molecule seems to be excluded from the enzyme annulus.

In a previous work (Castuma & Brenner, 1986a), the "in vitro" cholesterol enrichment of microsomal membranes was investigated from the point of view of the kinetics of UDP–glucuronyltransferase and the dynamic properties of the lipid bilayer. Taking into account differential cholesterol–phospholipid interactions (Guyer & Bloch, 1983) and the kinetic behavior of the reconstituted enzyme (Hochman et al., 1983), the results were rationalized on the basis that the sterol incorporation, while increasing the bulk lipid packing, fluidizes the UDP–glucuronyltransferase microenvironment. (Castuma & Brenner, 1986b). Yet, it remained to be proved whether this effect was enzyme specific or reflected a more general mechanism for the cholesterol regulation of microsomal proteins.

ESR spectroscopy is a particularly suitable technique to be used to answer the above question. It has be shown that the spectra of spin-labeled membranes can exhibit two components: a motionally restricted one, ascribed to lipids in the immediate environment of integral proteins, and a more mobile component, due to the bilayer lipids. Appropriate computer subtractions allow the separation and the qualitative and quantitative characterization of each constituent. (Devaux & Seigneuret, 1985; East et al., 1985; Silvius et al., 1984; Ryba et al., 1987).

In the present paper normal and cholesterol-enriched microsomes (Castuma & Brenner, 1986a), and their extracted lipids, were spin-labeled with fatty acid and sterol derivatives. In this way, it was possible to examine the effect of cholesterol on the motional properties of lipids in the bulk fluid phase and at the protein interface of microsomal membranes.

† C.E.C. and R.R.B. are members of the Career of the Investigator of CONICET, Argentina. S.S. and M.T.L.F. are recipients of CNPq research fellowships. This work was supported by the CONICET-CNPq binational program and by research grants from FAPESP, FINEP, CNPq, and CONICET.

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0006-2960/91/0430-9492$02.50/0 © 1991 American Chemical Society

Materials and Methods

In Vitro Cholesterol Enrichment of Liver Microsomes. Cholesterol was incorporated into guinea pig liver microsomes as previously described (Castuma & Brenner, 1986a), by incubating microsomes with cholesterol-enriched vesicles prepared with extracted microsomal lipids. The latter were obtained from normal endoplasmic reticulum membranes following Folch's procedure (Folch et al., 1957), and the corresponding phospholipid fraction was separated in a silicic acid column eluted with methanol. To prepare cholesterol-enriched vesicles, an appropriate amount of the sterol was added to the phospholipid extract to reach a cholesterol/phospholipid molar ratio of 1:1. Butylated hydroxytoluene was added (0.25% w/w) to avoid lipid peroxidation. After the solvent was evaporated, 0.25 M sucrose pH 7.0 was added (final concentration 8 mM in phospholipids), and the mixture was sonicated for 20 min and centrifuged for 2 h at 120000*g* to discard large multilamellar vesicles, essentially according to Barenholz et al. (1977). All procedures were carried out at 3–5 °C under N_2 atmosphere. These vesicles were incubated with the 12000*g* supernatant for 1 h and centrifuged at 105000*g* to obtain the cholesterol-enriched microsomes (Castuma & Brenner, 1986a). The integrity of microsomal membranes was checked by measuring mannose-6-phosphatase latency according to Arion et al. (1980).

Lipids from these modified microsomes were extracted and separated as mentioned above. Inorganic phosphate and cholesterol were quantitated according to the procedures of Chen et al. (1956) and Huang et al. (1961), respectively. The protein content was assayed by the method of Lowry et al. (1951).

Spin Labeling of Samples and ESR Measurements. Stock 1 mM solutions of 5-, 12-, and 16-SASL[1] and ASL were prepared in chloroform and stored at −80 °C. To label the different microsomal or lipid suspensions, appropriate amounts of the labels were dried under N_2, and the membrane suspensions were added and gently agitated for 10 min at low temperature (4–6 °C). The preparations were used for ESR measurements within 3 h. In all cases, the final lipid concentration was 10 mM and the molar ratio lipid/label was 150:1 or higher, to prevent spin exchange between probe molecules.

ESR measurements were performed in a Bruker ER 200D-SRC spectrometer. The temperature was controlled to about 0.5 °C with a Bruker B-ST 100/700 variable temperature device. Spectra were recorded with phase-sensitive detection at 100 kHz and stored and manipulated in an Aspect 2000 computer system. A total of 2048 data points were taken for a scan width of 100 G. The temperature was scanned from 5 to 40 °C and monitored with a Bailey digital thermometer with a thermocouple probe positioned outside the sample cell in the center of the cavity.

Spectral analysis was performed as described by Esmann et al. (1985), with a single-component subtraction procedure. The subtracted spectrum may correspond either to the motionally restricted or to the mobile population. In our experiments the former was chosen from a library of spectra of DMPC or egg PC, with or without cholesterol, at low temperatures, and the latter was obtained from the extracted microsomal lipids. The criteria for normalization of spectra included an average value of almost zero for the whole spectrum, values of zero at the start and end of the single integral, and a double integral with no negative slopes.

Effective order parameters, S_{eff}, were calculated from the expression

$$S_{eff} = \frac{A_\parallel - A_\perp}{A_{zz} - (1/2)(A_{xx} + A_{yy})} \frac{a_0'}{a_0}$$

where $2A_\parallel$ is the maximum hyperfine splitting and

$$A_\perp = A_{min} + 1.4\left[1 - \frac{A_\parallel - A^{min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\right]$$

where $2A_{min}$ is the inner hyperfine splitting (Griffith & Jost, 1976; Gaffney, 1976). The polarity was normalized from a_0'/a_0, where

$$a_0' = (1/3)(A_{xx} + A_{yy} + A_{zz})$$

and

$$a_0 = (1/3)(A_\parallel + 2A_\perp)$$

A_{xx}, A_{yy}, and A_{zz} being the principal values of the hyperfine tensor for doxylpropane (Griffith & Jost, 1976).

Effective correlation times, τ_{eff}, for the motionally restricted labels were calculated by the Freed empirical expression (Freed, 1976), assuming Brownian motion for the acyl chains

$$\tau_{eff} = a\left(1 - \frac{A_\parallel}{A_{zz}}\right)^b$$

where $a = 5.4 \times 10^{-10}$ s and $b = -1.36$, in the slow-motion regime.

For the bulk fluid phase labels, correlation times were calculated by assuming pseudoisotropic motion based on the expression (Schreier et al., 1978)

$$\tau_B = -1.22 \times 10^{-9} B$$

where

$$B = (1/2)\Delta H(0)[(h_0/h_{+1})^{1/2} - (h_0/h_{-1})^{1/2}]$$

$\Delta H(0)$ is the central line width, and h_m are the line heights for the m = 1, 0, and -1 lines.

Results

The "in vitro" cholesterol incorporation increased the sterol content from 18% in native microsomes to 33% in modified membranes (mol % relative to total lipids). Values for mannose-6-phosphatase latency were 85% and 83% respectively, indicating no loss of membrane integrity due to the incubation procedure.

The temperature dependence of the ESR spectra of ASL and 12-SASL labeled normal microsomes and their extracted lipids are shown in Figure 1. The spectra of native membranes (left column) exhibit two components, one corresponding to a normal fluid bilayer and the other to more motionally restricted lipids, usually ascribed to those interacting directly with the intramembranous surface of proteins. The three narrow peaks at the central region of the spectra correspond to the fluid component signal, which is similar to the spectra of extracted lipids (right column). The spectrum of the motionally restricted component is broader than that of the fluid bilayer and is resolved in the outer wings of the latter (indicated by the arrows in Figure 1). The difference in line width for the two components for a wide temperature range illustrates the difference in chain rotational dynamics in the two lipid environments. Line width measurements show that the fluid component has a much steeper temperature depen-

[1] Abbreviations: *n*-SASL, *n*-(4,4-dimethyl-3-oxy-2-oxazolidinyl)-stearic acid; ASL, 17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxy; ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene.

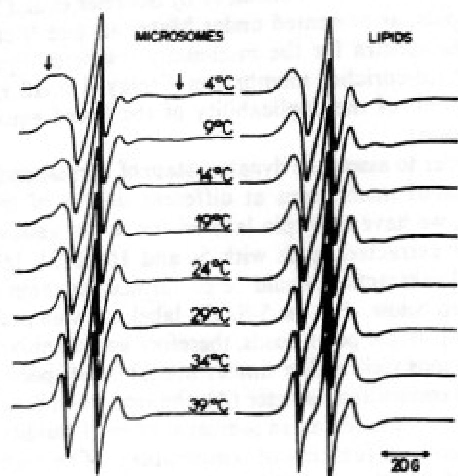

FIGURE 1: Temperature dependence of ESR spectra of 12-SASL-labeled normal microsomes and lipids. The arrows indicate the motionally restricted component. Total scan width = 100 G.

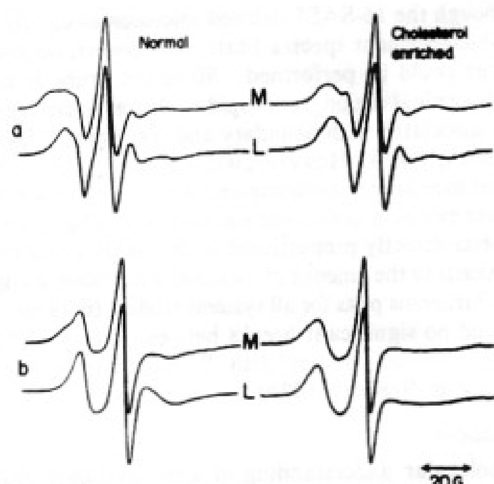

FIGURE 2: Spectra of normal and cholesterol-enriched microsomes (M) and their extracted lipids (L), labeled with 12-SASL (a) and ASL (b).

dence than the motionally restricted component.

Figure 2a shows the 12-SASL spectra of normal and cholesterol-enriched microsomes together with the corresponding extracted lipids, at 4 °C. In this case, two-component spectra are clearly seen in normal microsomes. In cholesterol-enriched membranes, the two components are less resolved. The spectra, as well as spectral deconvolution (see below) indicate that the motionally restricted lipids display greater mobility in the cholesterol-enriched than in the normal membranes.

In agreement with the known effects of cholesterol on liquid crystalline phases (Schreier-Muccillo et al., 1973), the extracted lipids from modified membranes exhibit a greater spectral anisotropy than those from native microsomes, both for 12-SASL- and ASL-labeled bilayers (Figure 2a and b).

The spectra of ASL-labeled microsomes and extracted lipids are given in Figure 2b. The line shapes are rather different than those of 12-SASL, due to the rigid structure and different orientation of the hyperfine and g-tensors of this probe in the bilayer (Schreier et al., 1978). Nevertheless, the motionally restricted component is still seen in the low-field region of the microsome spectrum (M). For the cholesterol-enriched membranes, however, the broadening of the bilayer component and the narrowing and decrease in hyperfine splitting of the protein-bound component lead to a loss of resolution of the two components. Nevertheless, the existence of these components is clearly seen upon spectral deconvolution (see below). In addition, although the two components are not well resolved in the cholesterol-enriched microsomes at all temperatures studied, the spectra cannot be matched by any single-component spectra, being rather distinct from labeled extracted lipids enriched with different amounts of cholesterol, or even combinations of the latter. Moreover, subtraction of any lipid spectrum from the membrane spectra always yielded the spectrum of a more motionally restricted component, as described below.

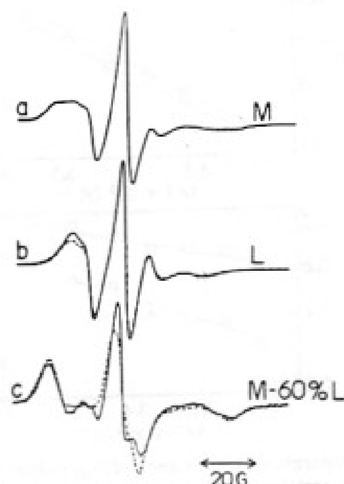

FIGURE 3: Spectral deconvolution for 12-SASL-labeled normal microsomes at 4 °C. Normal microsomes (a, M). The full lines correspond to the extracted microsomal lipids (b, L) and to M minus 60% L (c). This subtraction yields the spectrum corresponding to the motionally restricted lipids. The spectrum of egg PC-50 mol % cholesterol (dashed line, c) was chosen to represent the motionally restricted component (see text). Subtraction of this spectrum from M yields that corresponding to the fluid bilayer lipids. The resulting spectrum is given in panel b, dashed line.

The quantitative evaluation of the composite spectra was performed by digital subtraction, as previously described (East et al., 1985; Ryba et al., 1987).

Subtraction was effected by two different procedures, either by subtracting the spectra corresponding to the mobile component or by doing the same with the motionally restricted component. In the first case, we used spectra of the extracted lipids, at the appropriate temperature. In the second case, a library was constructed with a variety of lipid systems, at different temperatures. Spectra for subtraction were chosen so as to match the outer extrema of the experimental composite spectra.

The results are illustrated in Figure 3 for 12-SASL-labeled microsomes (Figure 3a) and their extracted lipids (Figure 3b, full line) at 4 °C. For subtraction of the motionally restricted component, we chose the spectra of egg PC-50 mol % cholesterol vesicles, at 28 °C. This spectrum is given in Figure 3c, dashed line. The subtraction of this spectrum from that in Figure 3a yields the fluid component (Figure 3b, dashed line), which corresponds closely to that of the extracted lipids at 4 °C (Figure 3b, full line). The end point of the subtraction was most critically judged by the absence of a negative contribution in the low field shoulder of the resultant more fluid[2] spectrum.

Complementary subtraction of the lipid spectrum (Figure 3b, full line) from the membrane spectrum (Figure 3a) yields the spectrum of the motionally restricted component (Figure

[2] The term "fluidity" is used throughout this work as an operational parameter including contribution from both rate and amplitude of motions.

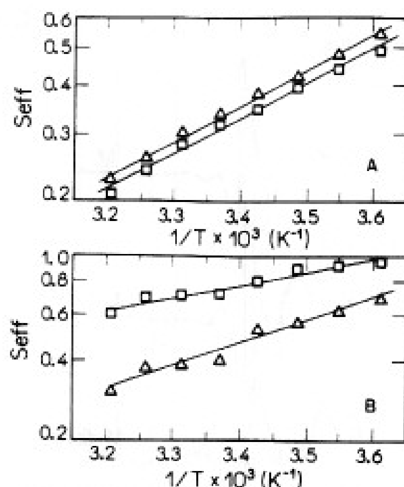

FIGURE 4: Temperature dependence of S_{eff} values for the fluid (A) and motionally restricted (B) component of normal (□) and cholesterol-enriched (Δ) microsomes.

3c, full line), which corresponds very closely to the motionally restricted reference spectrum used before (Figure 3c, dashed line). Double integration gives the fractions of the components present in the composite spectrum. The two independent methods of quantitation agree within ±4%.

The above subtraction procedure works well at low temperatures. At higher temperatures, however, the resolution of the microsome spectra is rather difficult, because the two components are coupled by slow exchange (East et al., 1985). In this respect Ryba et al. (1987) demonstrated that the effects of exchange can be satisfactorily simulated by choosing individual spectral components that best match the apparent components in the exchange-coupled spectrum. Accordingly, in the present work, the fluid component was taken from the extracted lipids recorded at the necessary lower temperature.

The fraction of the motionally restricted component (f) was found to be 0.42 ± 0.05 for both 12-SASL- and ASL-labeled microsomes. There was no significant temperature dependence. In addition, no significant difference in f was found between normal and cholesterol-enriched membranes.

Spectral subtractions as shown in Figure 3 also allow analysis of the structural/dynamic properties of the two lipid components in terms of effective order parameters (S_{eff}). The S_{eff} values, calculated as described under Materials and Methods, can only be considered as apparent values, since their derivation assumes the fast motional limit (Freed, 1976), and the spectra presented here probably contain contributions from slow molecular motions. This contribution is particularly important for low-temperature spectra and for the motionally restricted component, where the probe is more immobilized due to its contact with integral membrane proteins. However, S_{eff} values are useful as a measure of the degree of order and/or motional restriction of the probes.

Figure 4 shows the temperature dependence of S_{eff} for the components of 12-SASL-labeled systems. These data confirm the qualitative analysis made before (Figure 2): cholesterol incorporation, while increasing the S_{eff} values of the fluid component, decreases those of the motionally restricted component, as postulated by Castuma et al. (1986a,b). In addition, the Arrhenius plots of Figure 4 reveal no discontinuities in either the fluid or motionally restricted component, indicating the absence of a phase transition along the temperature range studied.

Similar profiles were obtained for effective correlation times (τ_{eff}) calculated as described by Freed (1976), for the motionally restricted component, or by Schreier et al. (1978), for bulk lipids, as presented under Materials and Methods. In fact, the spectra for the motionally restricted lipids in the cholesterol-enriched membranes displayed small τ_{eff} values, in the limit of the applicability of the Freed equation (see Discussion).

In order to assess the dynamic state of normal and modified microsomal membranes at different depths of the bilayer matrix, we have also spin labeled the intact vesicles as well as their extracted lipids with 5- and 16-SASL labels. No spectral subtractions could be performed for them as will be explained below. For the 5-SASL label, the spin probe is near the phospholipid polar heads, therefore both annular and bulk lipid regions yield rather similar immobilized spectra. In this case the empirical parameter (Δ), the separation between outer extrema, was taken as an indicator of lipid fluidity and was measured as a function of temperature. The higher values obtained for the composite spectra, for both normal and modified microsomes, compared to those of the extracted lipids can be ascribed to the annular lipids present in the native membranes.

Although the 16-SASL-labeled microsomes clearly exhibit the two-component spectra (data not shown), no good subtractions could be performed. Since the probe is near the methyl-terminal region, very rapid exchange averages the line shapes associated with boundary and free lipids. (Devaux & Seigneuret, 1985). However, when normal and cholesterol-enriched membranes are compared with their extracted lipids, the latter exhibit a noticeable increase in h_{+1}/h_0, an empirical parameter directly proportional to the fluidity and inversely proportional to the amount of motionally restricted component.

The Arrhenius plots for all systems studied (data not shown) evidenced no significant breaks between 4 and 40 °C, confirming previous results with fluorescence spectroscopy (Castuma & Brenner, 1989).

Discussion

A molecular understanding of any functional biological membrane requires the knowledge of how the hydrophobic regions of proteins are interfaced with the fluid lipid bilayer. The proteins seem to be surrounded by a somewhat spatially disordered lipid arrangement, superimposed with an on–off exchange between boundary lipids and the fluid bilayer matrix (Devaux & Seigneuret, 1985; Marsh & Watts, 1982, 1988).

Most ESR studies have been performed with purified enzymes reconstituted with lipids of defined composition (Marsh & Watts, 1988). Yet, a small amount of work has been done employing spin labels to monitor lipid–protein interaction in heterogeneous natural membranes (Bigelow et al., 1986, Li et al., 1989).

The endoplasmic reticulum is one of the main eukaryotic membranes where many central metabolic reactions take place (Brenner, 1974). In this regard, the present work examines lipid–protein interactions in the microsomal membrane and, particularly, the cholesterol-induced lipid reorganization in the microsomal protein microenvironment.

In the present work, lipid–protein interaction in microsomal membranes was monitored by ESR spectroscopy, similarly to what has been done for other biological membranes (Marsh & Watts, 1988). Namely, a motionally restricted component is observed in 12-SASL- and ASL-labeled microsomes in addition to the fluid lipid component seen in dispersions of the extracted membrane lipids (Figures 1, 2, and 3).

Cholesterol enrichment of native membranes provides evidence that the sterol modulates lipid–protein interactions in microsomes. This modulation is characterized by two prop-

erties: (1) cholesterol enrichment does not affect the proportion of the motionally restricted component in both 12-SASL- and ASL-labeled membranes, suggesting that energies associated with phospholipid–protein and phospholipid–cholesterol interactions are of similar magnitude; and (2) in contrast with the well-known rigidifying effect of cholesterol on bilayer lipids (Schreier-Muccillo et al., 1973), also seen in the present work (Figure 4), the spectral line shapes indicate a striking sterol-promoted increase in the mobility of the motionally restricted labels.

The rationale behind our results depends, however, on the applicability of some criticisms currently raised against the two-component model for lipid–protein interactions. The motionally restricted component has been suggested to be due to lipids trapped between proteins (Hoffmann et al., 1981). Still, as pointed out under Results, these lipids appear to exchange with bulk lipids, especially at higher temperatures, when the mobile component was matched by the extracted lipids at a somewhat lower temperature than the corresponding membranes. In addition, the fraction of constrained lipids remains essentially constant over a large temperature range. Therefore, microsomal proteins do not seem to undergo any change in their aggregation state since this would be expected to alter the exposed protein surface and consequently the amount of annular lipids.

Silvius et al. (1984) have proposed that the motionally restricted component could arise from a specific binding of fatty acid spin labels, not necessarily at the intramembranous surface of proteins. This does not seem to be the case for our system, since the motionally restricted component is observed with both ASL and 12-SASL. To check whether the spin labels would be occupying available sites at the cytoplasmic surface of the protein, microsomes were incubated with stearic acid before labeling. Neither the amount nor the nature of the motionally restricted component were affected. On the other hand, we have incorporated spin-labeled phosphatidylcholine in microsomes and their extracted lipids (results not shown) and performed the corresponding subtractions. This analysis reveals that, although the quantitative results are not the same as those obtained with stearic acid, the spectral parameters also indicate an increase in the fluidity of the motionally restricted component upon cholesterol incorporation.

τ_{eff} values calculated for 12-SASL spectra fall between 4×10^{-9} and 1×10^{-9} s for the fluid component; whereas, for the motionally restricted one, τ_{eff} values lie between 10^{-7}–10^{-8} s and $(15–3) \times 10^{-9}$ s, for the native and cholesterol-enriched systems, respectively, for the temperature range studied. The subtracted spectra obtained at higher temperatures probably do not reflect the actual order/mobility of the labels, since at these temperatures the two populations are very likely coupled by slow exchange, with a frequency of 10^{-7} s^{-1} (Davoust & Devaux, 1982; East et al., 1985; Ryba et al., 1987).

In recent years, there have been conflicting results in the literature regarding the involvement of cholesterol in lipid–protein interactions (Klapauf & Schubert, 1977; Yeagle et al., 1988) and particularly in membrane protein functions [for a review, see Yeagle (1985)]. The sterol molecule was seen to stimulate, inhibit or not affect a number of protein-modulated activities in different systems. In the light of the current knowledge about this subject, some hypotheses could be put forward to explain our results. The finding that the protein-immobilized lipids in cholesterol-enriched membranes are more fluid than those in the native microsomes could mean that the annular lipids in the former system have a lower degree of order and/or rigidity. This effect could be ascribed to a partial segregation of unsaturated PC's toward the enzyme microenvironment, due to the preferential interaction of cholesterol with saturated PC's, as postulated in Castuma and Brenner (1986a,b) in previous works. This hypothesis has support in a number of results obtained with model systems. Cholesterol preference for saturated PC's has been found in lipid bilayers. Guyer and Bloch (1983) have shown that cholesterol preferably rigidifies (i.e., interacts with) bisaturated PC's, rather than unsaturated ones. In addition, Fugler et al. (1985) found that cholesterol interactions involving saturated chains are stronger than those with unsaturated phospholipids. These effects can be interpreted on the basis of steric factors that make the direct contact between cholesterol and unsaturated acyl chains less favorable.

Work with spin labels has also provided evidence for the cholesterol preference for saturated PC's in lipid bialyers. Kusumi et al. (1986), using phospholipid and stearic acid labels, demonstrated that the ordering effect of the sterol molecule is less marked in unsaturated PC molecules than in the saturated analogues. In a recent paper, the same authors (Pasenkiewicz-Gierula et al., 1990) provided a detailed analysis of the effect of acyl chain degree of unsaturation on the cholesterol–phosphatidylcholine interaction, as monitored by ASL. These results again indicated the cholesterol preference for saturated PC's.

It is interesting to note that Silvius et al. (1984) found that cholesterol increased the activity of the Ca^{2+}, Mg^{2+}-ATPase when the enzyme was reconstituted with DMPC, while the opposite occurred when the lipid was DOPC. These results could be rationalized in terms of a differential interaction of cholesterol with saturated and unsaturated phospholipids, which would be reflected in the lipid–protein interaction, giving rise to the different effects on enzyme kinetics.

Another possible explanation for our results would be a cholesterol-promoted increase in the exchange rates between boundary and bulk phase lipids (Ryba et al., 1987). In order to give further insight for this hypothesis, we have simulated the spectrum of cholesterol-enriched microsomes using the exchange model (Davoust & Devaux, 1982; East et al., 1985). The bulk lipid component was assumed to be the spectrum of the corresponding extracted lipids, and for the annular lipids we used the spectrum of the motionally restricted population in native microsomes. The simulated composite spectra using different exchange frequencies do not satisfactorily match the experimental ones, suggesting that the increase in the exchange rate between annular and bulk lipids cannot account for the observed differences between modified and native microsomes. Furthermore, exchange frequencies in the range of the ESR time scale (Figure 2) would be rather unexpected at 4 °C (East et al., 1985). ESR (Schreier-Muccillo et al., 1973; Mailer et al., 1974) and dynamic fluorescence depolarization (Kawato et al., 1978) measurements have shown that cholesterol increases the order of fluid phase PC bilayers while decreasing the mobility of acyl chains. In contrast, Straume and Litman (1987) found that rotational dynamics of DPH were accelerated in PC vesicles containing 30 mol % cholesterol. However, further work of the same authors (Straume & Litman, 1988) revealed that the incorporation of rhodopsin in the PC–cholesterol vesicles abolished the sterol-dependent acceleration of the probe depolarization rates, indicating a weak validity for the postulated exchange hypothesis.

Finally, Hochman et al. (1981) have shown that the kinetic behavior of microsomal UDP–glucuronyltransferase is similar in the native membranes and in the reconstituted system

containing gel-phase phospholipids. Thus, if the protein environment consisted of gel-phase-like lipids, regarding the mobility of the hydrocarbon chains, cholesterol incorporation above normal levels might increase the mobility of annular lipids. This rationale involves the assumption that the sterol molecule can enter the protein microenvironment, which is still a subject of discussion (Yeagle, 1985).

Whichever would be the explanation for our findings, the present work suggests that cholesterol incorporation, besides modifying bilayer properties, can influence the chemical and/or physical properties of annular lipids, which, in turn, would modulate protein function. We are currently investigating the cholesterol effects on reconstituted systems containing purified microsomal proteins.

ACKNOWLEDGMENTS

We are grateful to Dr. A. Watts and Dr. A. G. Lee for helpful discussions and for making the ESR simulation programs available.

Registry No. Cholesterol, 57-88-5.

REFERENCES

Arion, W. J., Lange, A. J., & Walls, A. E. (1980) *J. Biol. Chem. 255*, 10387–10395.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2811.

Bigelow, D. J., Squier, T. C., & Thomas, D. D. (1986) *Biochemistry 25*, 194–202.

Bloj, B., & Zilversmit, D. B. (1982) *J. Biol. Chem. 257*, 7608–7614.

Brenner, R. R. (1974) *Mol. Cell Biochem. 3*, 41–52.

Castuma, C. E., & Brenner, R. R. (1986a) *Biochemistry 25*, 4733–4738.

Castuma, C. E., & Brenner, R. R. (1986b) *Biochim. Biophys. Acta 855*, 231–242.

Castuma, C. E., & Brenner, R. R. (1989) *Biochem. J. 258*, 723–731.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Davoust, G., & Devaux, P. F. (1982) *J. Magn. Reson. 48*, 475–494.

Devaux, P. F., & Seigneuret, M. (1985) *Biochim. Biophys. Acta 822*, 63–125.

East, J. M., Melville, D., & Lee, A. G. (1985) *Biochemistry 24*, 2615–2623.

Esmann, M., Watts, A., & Marsh, D. (1985) *Biochemistry 24*, 1386–1393.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Freed, J. H. (1976) in *Spin Labeling, Theory and Applications* (Berliner, L. J., Ed.) Vol. I, pp 53–132, Academic Press, New York.

Fugler, L., Clejan, S., & Bittman, R. (1985) *J. Biol. Chem. 260*, 4098–4102.

Gaffney, B. J. (1976) in *Spin Labeling, Theory and Applications* (Berliner, L. J., Ed.) Vol. I, pp 567–571, Academic Press, New York.

Griffith, O. H., & Jost, P. C. (1976) in *Spin Labeling, Theory and Applications* (Berliner, L. J., Ed.) Vol. I, pp 454–523, Academic Press, New York.

Guyer, W., & Bloch, K. (1983) *Chem. Phys. Lipids 33*, 313–322.

Hochman, Y., Kelley, M., & Zakim, D. (1983) *J. Biol. Chem. 258*, 6509–6516.

Hoffman, W., Pink, D. A., Restall, J., & Chapman, D. (1981) *Eur. J. Biochem. 114*, 585–589.

Houslay, M. D., & Stanley, K. K. (1982) *Dynamics of Biological Membranes*, Wiley, New York.

Huang, T. C., Chen, C. P., Wefler, V., & Raftery, J. (1961) *Anal. Chem. 33*, 1405–1406.

Jones, O. T., & McNamee, M. G. (1988) *Biochemistry 27*, 2364–2374.

Kawato, S., Kinosita, K., & Ikegami, A. (1978) *Biochemistry 17*, 5026–5031.

Klappauf, E., & Schubert, D. (1977) *FEBS Lett. 80*, 423–425.

Kusumi, A., Subczynski, W. K., Pasenkiewicz-Gierula, M., Hyde, J. S., & Mekrle, H. (1986) *Biochim. Biophys. Acta 854*, 307–317.

Li, G., Knowles, P. F., Murphy, D. J., Nishida, I., & Marsh, D. (1989) *Biochemistry 28*, 7446–7452.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mailer, C., Taylor, C. P. S., Schreier-Muccillo, S., & Smith, I. C. P. (1974) *Arch. Biochem. Biophys. 163*, 671–678.

Marsh, D., & Watts, A. (1982) in *Lipid Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. 2, pp 53–126, Wiley-Interscience, New York.

Marsh, D., & Watts, H. (1988) in *Lipid Domains and the Relationship to Membrane Function*, pp 163–200, Alan R. Liss, New York.

McIntyre, J. O., Samson, P., Brenner, S. C., Dalton, L., Dalton, L., & Fleischer, S. (1982) *Biophys. J. 37*, 53–56.

Pasenkiewicz-Gierula, M., Subczynski, W., & Kusumi, A. (1990) *Biochemistry 29*, 4059–4069.

Ryba, N. J. P., Horvâth, L. I., Watts, A., & Marsh, D. (1987) *Biochemistry 26*, 3234–3240.

Schreier-Mucillo, S., Marsh, D., Dugas, H., Schneider, H., & Smith, I. C. P. (1973) *Chem. Phys. Lipids 10*, 11–27.

Schreier, S., Polnaszek, C. F., & Smith, I. C. P. (1978) *Biochim. Biophys. Acta 515*, 375–436.

Schroeder, F. (1984) *Prog. Lipid Res. 23*, 97–113.

Silvius, J. R., McMillen, D. A., Saley, N. D., Jost, P. C., & Griffith, O. H. (1984) *Biochemistry 23*, 538–547.

Straume, M., & Litman, B. J. (1987) *Biochemistry 26*, 5121–5126.

Straume, M., & Litman, B. J. (1988) *Biochemistry 27*, 7723–7733.

Yeagle, P. L. (1984) *J. Membr. Biol. 78*, 201–210.

Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.

Yeagle, P. L., Young, J., & Rice, D. (1988) *Biochemistry 27*, 6449–6452.