Biochimica et Biophysica Acta 939 (1988) 102–110
Elsevier

BBA 73911

Interactions between a paramagnetic analogue of cholesterol and filipin

Luc Maurin, Frédéric Bancel, Philippe Morin and Alain Bienvenüe

Laboratoire de Biologie Physico-Chimique (UA 530 CNRS), USTL, Montpellier (France)

(Received 29 September 1987)

Key words: Cholesterol analog; Filipin; ESR; Electron microscopy

A paramagnetic analogue of cholesterol (called 25-doxyl-27-norcholesterol (CNO)), labeled near the w-end of the hydrophobic tail, was used to study interactions of cholesterol with filipin. We observed by electron microscopy that CNO- and cholesterol-filipin complexes are structurally equivalent. Two kinds of complexes were seen by ESR spectroscopy and electron microscopy, depending on the stoichiometric *R* ratio between the antibiotic and sterol. When *R* was high, an immobilized ESR spectrum appeared, showing strong imbrication between CNO and filipin. When *R* was nearer to unity, an exchange-broadened spectrum emerged, corresponding to a new phase that was very rich in CNO (a fast exchange between spins could occur by nearest contacts). CNO was easily displaced from its complex (i) by gradual addition of genuine cholesterol; and (ii) by an excess of phospholipids, owing to the very poor affinity of CNO (and cholesterol, by extension) for filipin in the lipidic phase. Almost no difference appeared between the ESR spectra of oriented samples, i.e. the probe showed no long-range order in any complex of CNO with filipin.

Introduction

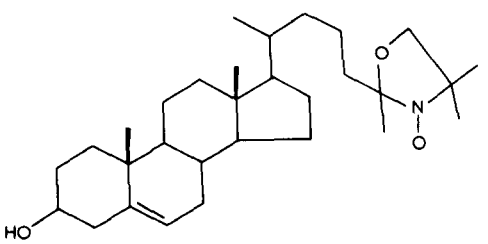
Cholesterol is an essential element in the functioning of mammal cells. In addition to the cases in which this compound is transformed (into steroid hormones, for example), it is also, directly involved in the structural stability of plasma membrane. However, many of its exact functions remain unknown. For instance, it is not understood how cholesterol, which is synthesized in endoplasmic reticulum, becomes concentrated in plasma membrane [1]. In somatic cells, for example, this membrane (which represents only 2% of total membrane) collects more than 80% of the cholesterol [2]. This property was determined on the one hand by assaying cholesterol in purified plasma membranes and on the other by electron microscopic observation of the appearance of about 20 nm particles when a polyene antibiotic, filipin, was added to cholesterol-containing membranes [3]. Another polyene antibiotic, amphotericin B, specifically interacts with these cholesterol-containing membranes, probably creating small diameter pores which can induce ion leakage and result in cell lysis [4–6]. This is one of the few examples of specific interaction between cholesterol and hydrophobic compounds, as there are a small number arguments in favour of specific interaction between cholesterol and membrane proteins [7,8].

Polyene antibiotic are consequently particularly appropriate for studying the structure of membrane complexes involving cholesterol. A number

Abbreviations: CNO, 25-doxyl-27-norcholesterol; FI, filipin; PC, egg yolk phosphatidylcholine; I, immobilized spectrum component; E, exchange-broadened component; F, fast mobile component.

Correspondence: P. Morin, Laboratoire de Biologie Physico-chimique (UA 530 CNRS), USTL, F-34060 Montpellier Cedex, France.

of papers have already dealt with the stoichiometry [9], the formation rate [10] and the structure of these complexes [11], as well as the specificity of the interaction between polyene antibiotics and various derivatives of cholesterol [6]. Results concerning the structure and the life times of the complexes remain ambiguous, although they have been obtained with many different methods [12–15]. In this paper, we describe results obtained by ESR spectroscopy on a novel paramagnetic analogue of cholesterol, designated here as CNO (25-doxyl-27-norcholesterol), whose structure is specified below. Two previous papers showed that CNO exactly mimics the behavior of genuine cholesterol in three domains: (i) its influence on the phase transition of phospholipids, (ii) the formation of pores in membranes in the presence of amphotericin B and their destabilization when filipin is added [16] and (iii) its interactions with cytochrome $P\text{-}450_{scc}$, an enzyme from the internal membrane of adrenocortical mitochondria that catalyzes the transformation of cholesterol into pregnenolone [25]. We demonstrate here that filipin acts essentially by excluding cholesterol from the membrane phase in a poorly organized complex.



Structure formula of CNO.

Materials and Methods

Chemical compounds. The synthesis of CNO has been described in a previous paper [16]. Egg yolk phosphatidylcholine and filipin were purchased from Sigma. Cholesterol was a generous gift from the Laboratory l'Oreal. These three compounds were used without any further purification. Buffer components were analytical grade from Merck.

ESR spectroscopy. Samples were prepared by removal of solvent in a vacuum, from a methanol solution containing the different compounds (phospholipids, polyene antibiotics, CNO, or cholesterol, depending on the experiment) and were suspended with a Vortex mixer in a 10 mM Tris, 200 mM NaCl buffer (pH 7.35). Samples then underwent several cycles of freeze-thawing (generally five) until the shape and amplitude of ESR spectra were stabilized. Spectroscopic measurements were performed in flat (0.2 mm) quartz cells with a thermoregulated device (± 1 °C) from Brucker. Oriented samples were obtained as described by Oelschlager et al. [17]. The ESR spectrometer was a Brucker ER 200D apparatus, interfaced with an Apple II + microcomputer. (1024 points are recorded on the X axis.) Further electronic processing of the ESR spectra (double integration, simulations, and subtracting) was done with home-made computer programs.

Electron microscopy. A liposome suspension was applied to the grids, flushed with a few drops of negative stain (uranyl acetate) and drained through filter paper. Specimens were photographed on a Jeol 200CX electron microscope.

Results

Electron microscopy of mixtures of phosphatidylcholine (PC), filipin and CNO (Fig. 1B) showed two patterns of complexes identical to those observed with genuine cholesterol [3]. When the ratio $R = (\text{filipin})/(\text{CNO})$ was very high (for instance about 70), broad complexes (approximate diameter of 100–180 nm) were visible (photo a), without any membrane-like structure. In contrast the, 'holey sheets' appeared together with bilayer structures for low values of R (for instance about of 5 or less) (photo b).

The ESR spectrum of CNO showed different components in the presence of filipin: Fig. 1A a and b shows ESR spectra obtained at 4 °C with the same solutions as in Fig. 1B. CNO in pure PC, under the conditions of Fig. 1A c, was fast rotating and produced a so called F typical spectrum. When the ratio $R = (\text{filipin})/(\text{CNO})$ was high, the resultant spectrum (Fig. 1A a) showed a characteristic shape, with a very long distance (66 gauss) between extrema, and was an immobilized typical spectrum (I). At low value of R, the spectrum (Fig. 1A b) had two components. The first with sharper peaks resembled the F type. The second

component shifted the whole spectrum above the base line at low field and below at high field: it consists thus in a single broad band, as observed in media where efficient spin exchange broadening occurred, and it was called the E type.

For intermediate values of R the three components, of types F, I, and E, occurred together in various proportions, as discussed below. For rather high values of R the proportions of I and F components was obtained by subtracting spectra of type I from the experimental spectrum and resulting in an F type. For low values of R, if we subtracted F type from the experimental spectrum (Fig. 1A b or Fig. 3 b), the result was not any reasonable spectrum of type E, because the free component was broader than the F type in pure PC. To evaluate the best shape of the two components, the following operations were carried out: different E spectra were obtained by simulation and subtracted from the experimental spectrum (Fig. 3b) until an F type resulted, that was consistent with a simple broadening of the experimental F type (Fig. 1A c). The best E and F spectra obtained by this method are shown in Fig. 3 c and d.

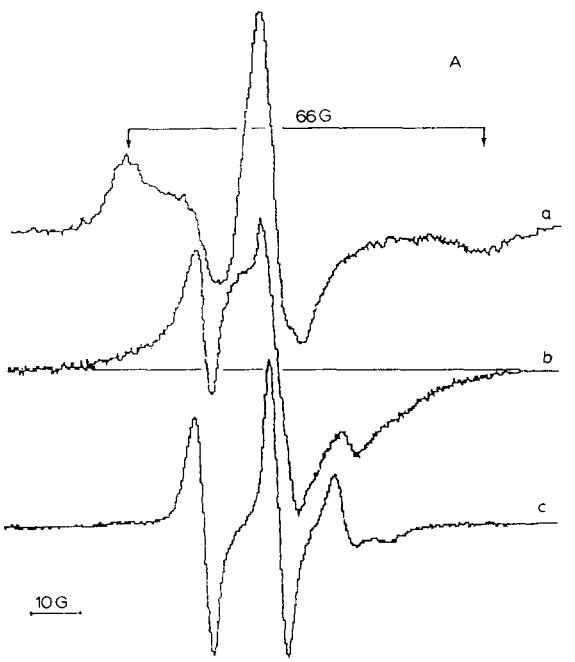
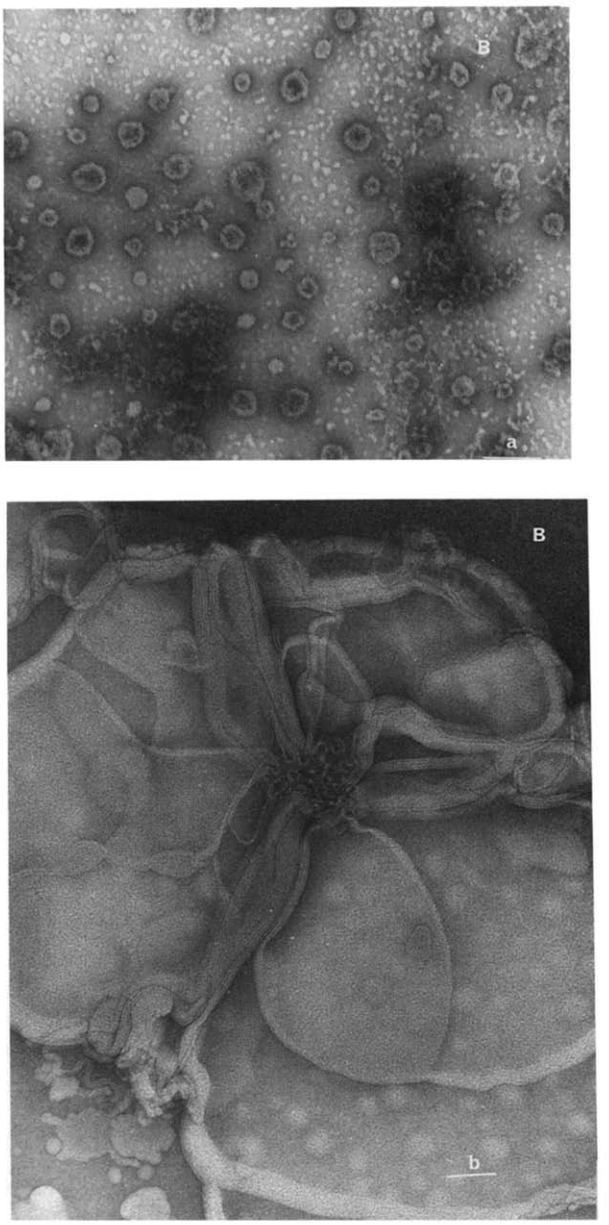


Fig. 1. (A) ESR spectra of liposome suspensions at 4° C. (a) PC (3 mM), FI (11 mM), CNO (0.16 mM). (b) PC (3 mM), FI (1.5 mM), CNO (0.3 mM). (c) PC (7 mM), CNO (0.03 mM). (B) Electron micrograph (negative staining) of samples; (a) bar: 230 nm and (b) bar: 90 nm.

Effect of diluting cholesterol and filipin in phospholipidic bilayers

The proportion of different spectral components depended on the concentration of the constituents in the hydrophobic phospholipid media.



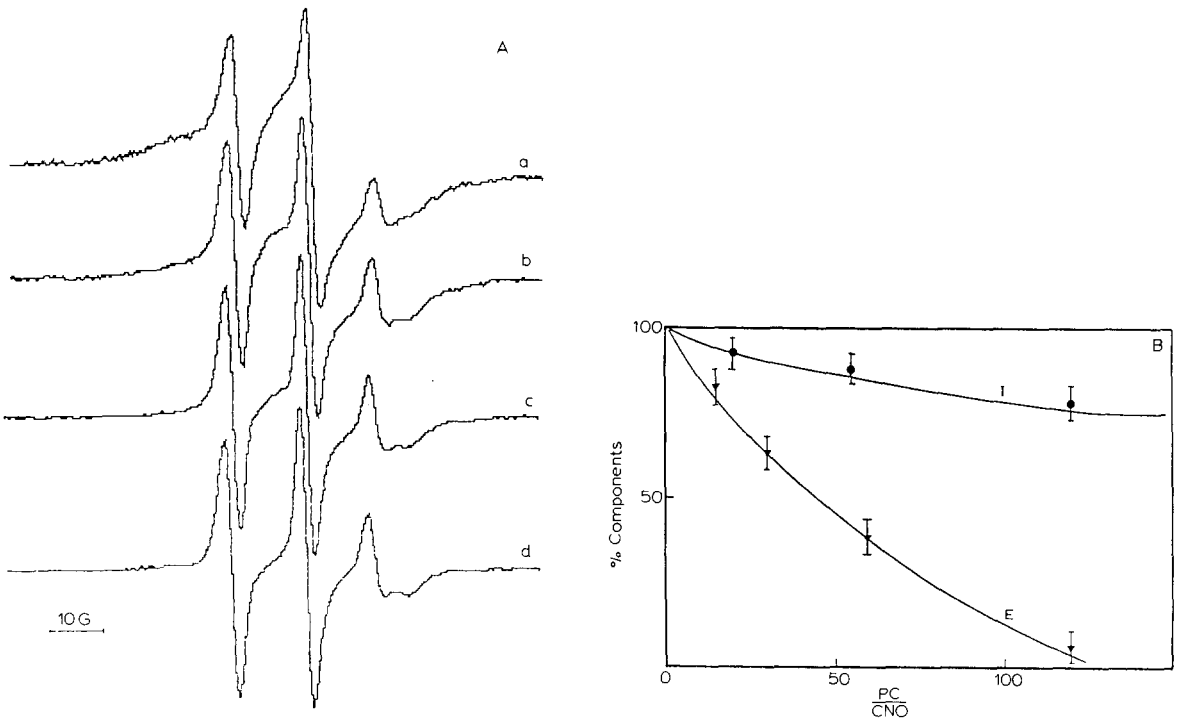


Fig. 2. (A) ESR spectra at 4° C, of samples containing PC (3 mM), FI (1.5 mM), CNO (0.3 mM), and various additions of PC: (a) 0, (b) 9 mM, (c) 18 mM, (d) 37 mM. (B) Evolution of immobilized (I) and exchange-broadened (E) components after displacement by PC. Initial samples had the following composition: PC = 3 mM, FI = 11 mM, CNO = 0.16 mM (●) and PC = 3 mM, FI = 1.5 mM, CNO = 0.3 mM (▼).

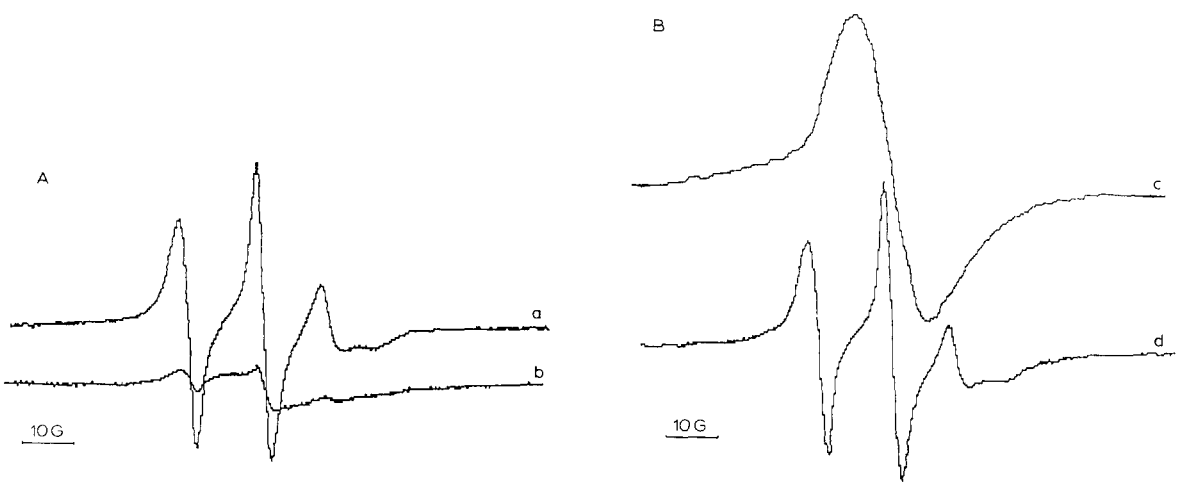


Fig. 3. ESR spectra (at the same amplification) of samples (PC = 7 mM, FI = 2 mM, CNO = 0.3 mM) after (a) and before (b) addition of cholesterol; (c) Simulated exchange-broadened spectrum used for subtraction; (d) free (F) component obtained by subtracting experimental ESR spectrum (b) from exchange broadened spectrum (c).

For example Fig. 2A shows that the F component increased when phospholipids were added at a fixed filipin/CNO ratio. Fig. 2B indicates the evolution of I and E proportions. Although the stoichiometry of the complexes filipin-CNO is not precisely known, the values of the apparent dissociation constant can be evaluated as follows:

$$C_t/CF = (K_{app}/F_t) + 1$$

where the concentrations, expressed as the molar fractions in the phospholipidic phase, are C_t, F_t and CF for total CNO, total filipin, and complex, respectively. The K_{app} values in the I and E case were 0.07 and 0.16, respectively.

Displacement by genuine cholesterol

Addition of cholesterol led to the disappearance of I and E components, as shown in Fig. 3 a, b. The intensity of the F component increased, as expected after displacement of CNO from its complex with filipin. The variation in the proportion of the different components can be plotted (Figs. 4 A–D) versus the amount of cholesterol added to the four initial samples, covering R values between 70 and 1. It should be noted that, in the absence of cholesterol, some of the spectra contained both I and E components in addition to an F component. The (F) form of fast rotating CNO increased as more cholesterol was added to the sample, until cholesterol displaced all

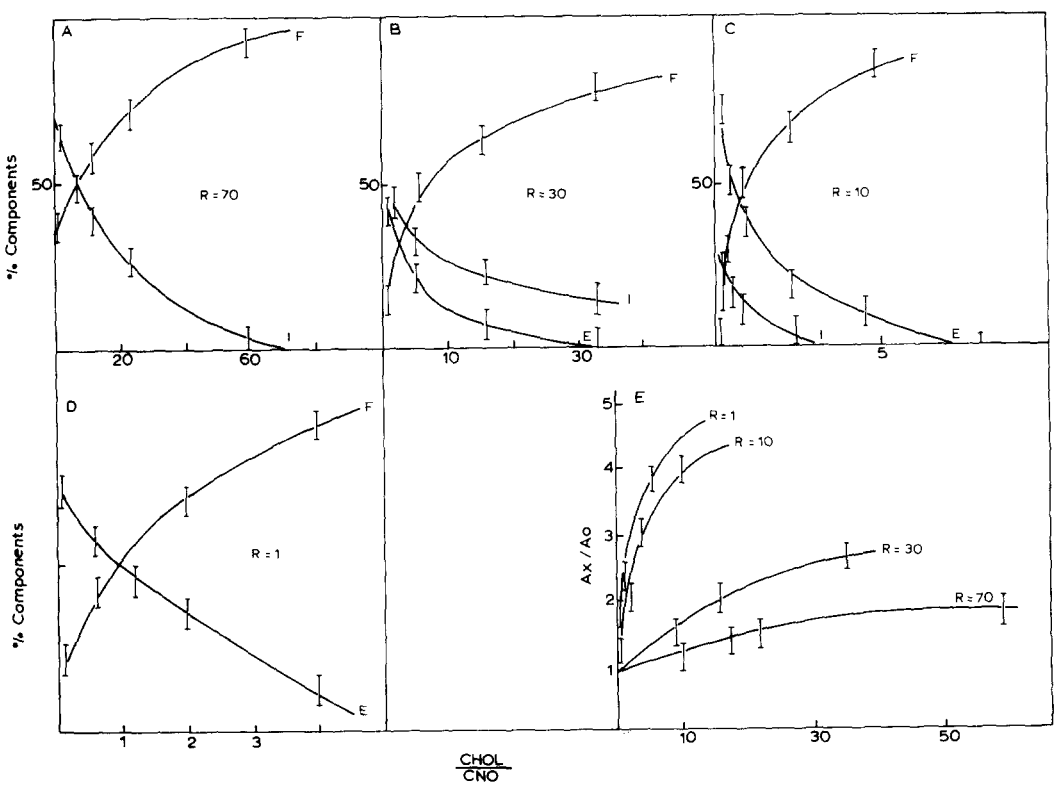


Fig. 4. Evolution in the proportions of different (I), (E) and (F) components, after addition of increasing amounts of cholesterol. Initial composition of liposomes were:
(A) PC = 7 mM, FI = 1.5 mM, CNO = 0.022 mM;
(B) PC = 7 mM, FI = 1.5 mM, CNO = 0.05 mM;
(C) PC = 7 mM, FI = 2 mM, CNO = 0.2 mM;
(D) PC = 1 mM, FI = 0.2 mM, CNO = 0.2 mM;
(E) evolution of central peak amplitude (relative) of samples A, B, C, and D. A_0 and A_x are signal heights (at constant gain) before and after addition of cholesterol.

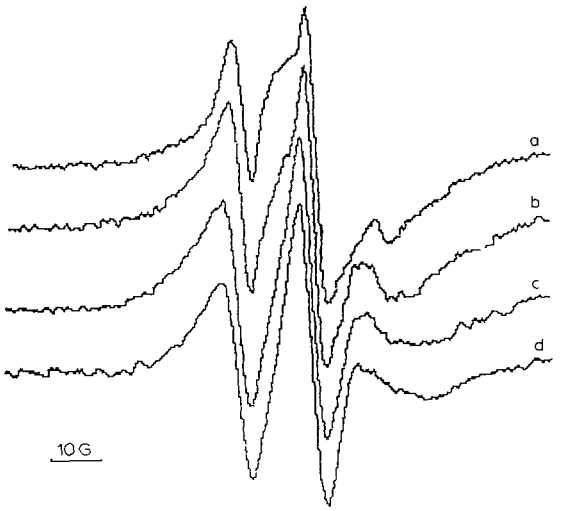


Fig. 5. ESR spectra obtained by dilution of CNO in complex. The PC, FI, and sterol (CNO + cholesterol) concentrations were kept constant (3 mM, 1.5 mM, and 0.3 mM, respectively). Cholesterol concentration: (a) 0 (b) 0.075 mM, (c) 0.15 mM, (d) 0.225 mM.

the CNO from its complex with filipin, at which point the R ratio was nearly one. Finally, the amplitude of the experimental spectrum (Fig. 4 E) increases saturably with the concentration of cholesterol, thus confirming the actual displacement.

The same proportions for E and I components were observed at 20 and 37 °C. A slight increase in the overall spectrum amplitude appeared but it was only due to the influence of temperature on the F component (data not shown).

Origin of the 'exchange broadened' E component

The observed E component could be due to dipolar interactions in the complex or to colission between probes [18]. To distinguish between these two causes, filipin, PC, and the ratio R' = (filipin)/(CNO + cholesterol) were maintained constant ($R' = 5$) in samples while the CNO/cholesterol ratio varied from 1:0 to 1:3. When the concentration of CNO was lowered by gradual dilution with cholesterol, the spin exchange was less efficient and E component vanished (Fig. 5). When the CNO/cholesterol ratio was 1:3 the spectrum appeared like an F type, which was somewhat broadened by spin exchange, since the ratio CNO/cholesterol was still high. If the filipin-CNO interactions had been the same at $R = 5$ as at $R = 70$ (Fig. 1A a), an I component would become visible as E faded. At higher temperature (20 and 37 °C), we observed the same results as at 4 °C (data not shown).

It can thus be concluded that CNO interacts much less strongly with filipin in E form than in the I form.

Oriented samples

In a macroscopically oriented sample arranged either in parallel of perpendicular to the magnetic field, the local order of the doxyl group can be determined by analysis of the ESR spectrum shape [19]. The shape variations of the oriented spectra obtained with samples placed on a quartz plate were very slight (Figs. 6A, 6B) both when the probe were immobilized (I form) and exchange broadened (E form). In contrast, the spectra obtained with oriented model membranes showed obvious differences depending on the orientation [16].

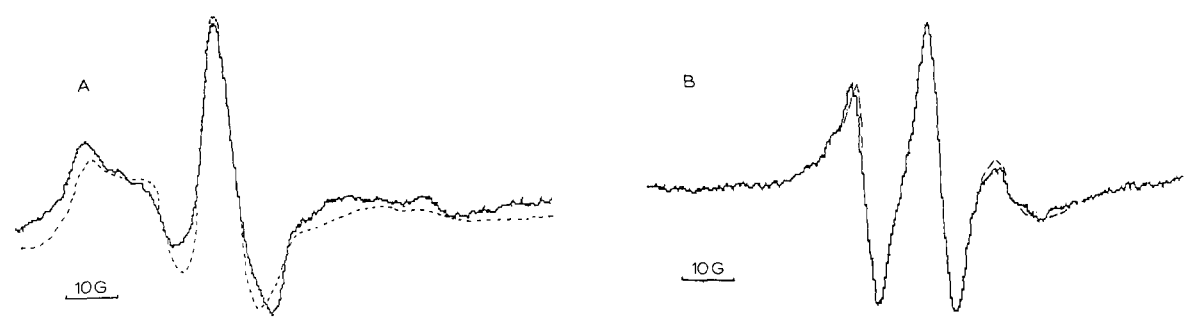


Fig. 6. ESR spectra of oriented egg phosphatidylcholine multilayers containing FI and CNO. The magnetic field was parallel (———) or perpendicular (- - - - -) to the supporting plate. (A) $R = 70$, same sample as in Fig. 1A a; (B) $R = 5$, same sample as in Fig. 5 d.

Discussion

We first consider the proposition that all the results reported above as well as the models discussed below, representing interactions between CNO and filipin, can be extended to genuine cholesterol. In addition to the arguments in Refs. 16 and 25, we rely on the symmetry between CNO and cholesterol in the results of the present work, as well as the structural identity of the complexes that filipin forms with cholesterol and CNO (Fig. 1B), as observed by electron microscopy.

As shown in Fig. 1A, filipin strongly influenced the immediate environment of the nitroxide probe fixed to the end of the lateral chain of cholesterol. In cases where filipin was in large excess ($R = 30$ or 70), an "immobilized" component mainly appeared, whose spectroscopic parameters are consistent with a correlation time of the probe movements higher than 10^{-7} s. It is therefore possible that filipin captures CNO, in a rather mobile environment including the hydrophobic tail. In other words, CNO (and by extension cholesterol itself) might be completely separated from the membrane phospholipids. When the ratio R decreased, a new component appeared, showing a very strong exchange interaction between the spins of the free radicals. There are different ways to dissociate the filipin-CNO complex. Addition of stoichiometric amounts of cholesterol (Figs. 3 and 4) or ergosterol (data not given), or of a large excess of phospholipids (Figs. 2A, B) is sufficient to displace the CNO into the lipidic phase. As expected, to induce a complete displacement of CNO a much smaller excess of cholesterol was needed (compare Figs. 2B and 4A). It is noteworthy that the complex was formed in a reproducible manner only after many freeze-thawing cycles. Likewise, after addition of cholesterol or phospholipids, the exchange between bound CNO and free cholesterol took several minutes, which is very long compared with the characteristic times of magnetic resonance. This result is important in the explanation of the cryofracture electron microscopy experiments performed to determine the cholesterol-containing areas of the plasma membrane [20], since it proves that a cholesterol exchange event is unlikely within the time the biological membrane was incubated with filipin, at least at low temperatures. Further ESR experiments in electron microscopy conditions (biological membranes rather than model systems, relative concentrations of filipin and cholesterol, temperature, fixation with glutaraldehyde, etc.) should be carried out to clarify this point.

Moreover, our results indicate that the affinity of filipin for cholesterol is very low: an apparent dissociation constant of 0.15 was calculated with molar fractions of reactants expressed relative to the lipidic phase, which was the actual reaction medium. A 10-fold dilution of these two reactants led to a complete displacement of equilibrium (Fig. 2B), which is in agreement with two other studies: (i) after addition of filipin to membranes [3], no complex could be detected by electron microscopy, under a threshold of 5% cholesterol in the membranes; (ii) similarly, a larger excess of filipin was needed to induce leakage of the internal contents of liposomes [16] when the proportion of cholesterol in the membranes was reduced.

In the case where R was close to one, the strong interaction observed in exchange broadened spectra (Fig. 1A, spectrum b and Fig. 2A) could be the result of one of two phenomena: either a short distance exchange (less than 0.5 nm), due to numerous random collisions, as occurs in a very fluid medium in which the probe is highly concentrated; or from dipolar interactions occurring over longer distances (up to 5–10 nm) in systems in which relative spin orientations show, in contrast, very few variations [21]. Under the same conditions of stoichiometry and temperature, Dufourc and Smith [19], using ^{2}H-NMR, concluded that filipin immobilizes the cholesterol ring at 4°C on a time scale of 10^{-5} s, which would also obviously apply to the shorter time scale of ESR. The results in Fig. 5 clearly show that the probe fixed at the end of the hydrophobic tail of cholesterol was, in fact, highly mobile at all temperatures. The broadening observed when CNO was more concentrated was probably due to collisions between probes; of course, the probability of collisions decreases with the proportion of CNO in the complexes fraction of sterols and with decreasing temperature. By using our results and those of Dufourc et al. [19] concerning the experiments at 4°C, the cholesterol rings were shown to be immobilized, whereas the nitroxide probe on

the hydrophobic tail was very mobile. Even in phospholipid/cholesterol bilayer, the cholesterol side chain-end movement is not completely elucidated [22,23]. Thus, the large difference between rings and side-chain mobilities observed in filipin-CNO complex is yet more difficult to interpret. This could be the result of the nitroxide labeling and/or the use of DMPC by Dufourc et al. [19] in contrast with egg PC in our experiments. Another possibility is that the cholesterol tail is actually much more mobile than the rings, as observed for nitroxide spin label covalently bound to proteins, when the spacer arm is sufficiently long [24]. In any case, all the results are consistent with a gathering of cholesterol in a phase containing no or very few phospholipids. The filipin participating in the complex does not seem to be deeply imbricated with cholesterol, although it stabilizes the phase.

It is still very difficult to imagine the molecular organization of the filipin-cholesterol complexes. A model was proposed by De Kruijff et al. [11] essentially on the basis of geometrical considerations. It consists of a crown arrangement of imbricated molecules of cholesterol and filipin in which the cholesterol molecules are parallel to the membrane plane. Dufourc and Smith [19] have criticized this model, considering that the observed exchange of cholesterol between its complexes with filipin and the rest of the membrane at temperatures over 10 °C is scarcely consistent with a different orientation of the cholesterol molecules in these two situations. A high degree of cholesterol organization in the complexes is in fact doubtful. In our work, the ESR spectrum of the CNO in the phase in which cholesterol was gathered by filipin, showed almost no variation in the orientation of the sample relative to the magnetic field (Fig. 6B). The most likely explanation, considering the good superposition of the two spectra obtained (Fig. 6B), is that the probe is organized randomly in complexes relative to the membrane plane. This does not mean that the cholesterol is completely disordered. On the contrary, it is possible that cholesterol molecules are well ordered perpendicular to the water/lipid interface, but because of the very small radius of the interface curvature, no long-range order appears. This model is in agreement with the funnel shape proposed by Behnke et al. [3], whose hole is clearly visible by electron microscopy (Fig. 1Bb). Nevertheless, not enough evidence is yet available to settle unambiguously the case of the complexes in which cholesterol is immobilized (Fig. 6A): neither ESR spectroscopy nor electron microscopy provide any indication in favor of a microscopic orientation of these complexes.

In conclusion, the results reported here concerning the ESR spectra of CNO in the presence of near the same amount of filipin are consistent with a model in which cholesterol is gathered by filipin in a nearly pure phase, with a stoichiometry close to unity. According to the model, the hydrophobic tail of cholesterol would be very mobile in this phase. However, in the presence of a large excess of filipin, the tail would show a correlation time longer than 10^{-7} s. Finally, there is no evidence supporting a long-range organization of the complexes cholesterol molecules.

Acknowledgements

The authors thank Roussel UCLAF S.A., for the generous gift of sterol used in CNO synthesis and Mr. J. Goodfellow for his assistance with the translation.

References

1 Lange, Y., Banno, Y. and Nozawa, Y. (1983) Cell Struct. Function 6, 301–312.
2 Cooper, R.A. (1978) J. Supramol. Struct, 8, 413–430.
3 Behnke, O., Tranum-Jensen, J. and Van Deurs, B. (1984) Eur. J. Cell Biol. 35, 189–199.
4 Blum, S.F., Shohet, S.B., Nathan, D.G. and Gardner, F.H. (1969) J. Lab. Clin. Med. 73, 980–987.
5 De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30–43.
6 Capuozzo, E. and Bolard, J. (1985) Biochim. Biophys. Acta 820, 63–73.
7 Yeagle, P.L. (1985) Biochim. Biophys. Acta 822, 267–287.
8 Seigneuret, M., Favre, E., Morrot, G. and Devaux, P.F. (1985) Biochim. Biophys. Acta 813, 174–182.
9 D. Kruijff, B., Gerritsen, W.J., Merlemans, A., Van Dyck, P.W.M., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 44–56.
10 Clejan, S. and Bittman, R. (1985) J. Biol. Chem. 260, 2884–2889.
11 De Kruijff, B. and Demel, A. (1974) Biochim. Biophys. Acta 339, 57–70.

12 Mazerski, J., Bolard, J. and Borowski, E. (1983) Biochem. Biophys. Res. Commun. 116, 520–526.
13 Bunow, M.R. and Lewin, I.W. (1977) Biochim. Biophys. Acta 464, 202–216.
14 Bittman, R. and Fischkoff, S.A. (1972) Proc. Natl. Acad. Sci. USA 69, 3795–3799.
15 Dufourc, E.J., Smith, I.C.P. and Jarrell, H.C. (1984) Biochim. Biophys. Acta 776, 317–319.
16 Maurin, L., Morin, P. and Bienvenüe, A. (1987) Biochim. Biophys. Acta 900, 239–248.
17 Oehlschlager, A.C. and Laks, P. (1980) Can. J. Biochem. 58, 978–985.
18 Smith, I.C.P. and Butler, K.W. (1976) in Spin Labelling, Theory and Applications (Berliner, L., ed.), pp. 411–451, Academic Press, New York.
19 Dufourc, E.J. and Smith, I.C.P. (1985) Biochemistry 24, 2420–2424.
20 Behnke, O., Tranum-Jensen, J. and Van Deurs, B. (1984) Eur. J. Cell Biol. 35, 200–215.
21 Scandella, C.J., Devaux, P.F. and McConnell, H.M. (1972) Proc. Natl. Acad. Sci. USA 69, 2056–2060.
22 Dufourc, E.J., Parish, E.J., Chitrakorn, S. and Smith, I.C.P. (1984) Biochemistry 23, 6062–6071.
23 Suckling, K.E. and Boyd, G.S. (1976) Biochim. Biophys. Acta 436, 295–300.
24 Davoust, J., Bienvenüe, A., Fellmann, P. and Devaux, P.F. (1980) Biochim. Biophys. Acta 596, 28–42.
25 Lange, R., Maurin, L., Larroque, C. and Bienvenüe, A. (1988) Eur. J. Biochem., in press.