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Interaction of the polyene antibiotic filipin with model and natural membranes containing plant sterols

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The interaction of the polyene antibiotic, filipin, with individual or mixed plant sterols (stigmasterol, sitosterol, campesterol and 24-methylpollinastanol) incorporated into large unilamellar vesicles (LUV) of soybean phosphatidylcholine (PC) as well as the filipin interaction with purified membrane fractions from maize roots containing these sterols was investigated by ultraviolet (UV) absorption and and circular dichroism (CD) spectroscopy. With both types of membrane preparation, dramatic changes in the UV absorption and CD spectra of the antibiotic were evidenced. When LUV containing stigmasterol, sitosterol and/or campesterol were incubated with low filipin concentrations (i.e., for filipin/sterol molar ratios (r_{st}) lower than 1), CD signal characteristic of the formation of filipin-sterol complexes were observed. At higher r_{st} values, the filipin–sterol interaction was shown to be in competition with a filipin–phospholipid interaction. With 24-methylpollinastanol-containing LUV, the filipin–phospholipid interaction was detected even at r_{st} values lower than 1, which suggests a lower affinity of filipin for this sterol and emphasizes the structural differences between Δ^5-sterols and 9β,19-cyclopropylsterols. With sterol-free soybean PC LUV, a filipin–phospholipid interaction could also be evidenced. With maize root cell membranes containing either Δ^5-sterols or 9β,19-cyclopropylsterols, CD spectra similar to those obtained in the presence of LUV having these sterols as components were observed. Thus, the protein component of the membranes does not appear to be an important feature.

Introduction

The molecular basis of the action of polyene antibiotics is not yet well understood in spite of the great deal of information gathered over many years. The biological effects of these antifungal agents are believed to result mainly from their ability to interact with membrane-bound sterols [1,2]. As a consequence of such an interaction, an increase in cell membrane permeability is often evidenced [1,2]. Among the polyene antibiotics, the use of the filipin complex (a mixture of four pentaenes [3]) as a probe for cholesterol in biological membranes has become widely adopted since

Abbreviations and chemical nomenclature: PL, phospholipids; PC, phosphatidylcholine; PA, phosphatidic acid; LUV, large unilamellar vesicles; CD, circular dichroism; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; fenpropimorph, (*R,S*)-4-[3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine; stigmasterol, (24*S*)-ethylcholesta-5,22*E*-dien-3β-ol; campesterol, (24*R*)-methylcholest-5-en-3β-ol; sitosterol, (24*R*)ethylcholest-5-en-3β-ol; 24-methylpollinastanol, 14α,(24*R*)- and 14α,(24*S*)-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol; cholesterol, 5α-cholesten-3β-ol; cycloeucalenol, 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; obtusifoliol, 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol.

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the demonstration in 1973 [4,5] that, in membranes containing 3β-hydroxysterols, filipin induced characteristic deformations or lesions which could be observed in freeze-fractured or negatively stained membranes [6–9]. However, because the molecular mechanism of the filipin-sterol complex formation is unknown, the feasibility of this probe for determining the exact distribution of sterols in membranes has to be questioned [8,9].

Many studies have been performed with either model or animal and fungal membranes. These studies indicate that filipin interacts specifically and stoichiometrically with sterols to give filipin-sterol complexes [1,2]. The structural features of sterol for an optimal interaction were shown to be a free 3β-hydroxyl group, a flat tetracyclic nucleus and an 8- to 10-carbon aliphatic hydrophobic side-chain [10,11]. Similar sterol structural requirements were reported to be involved in PC–cholesterol interactions [12,13], suggesting that, in the presence of filipin, a kind of competition might take place between the phospholipids and the antibiotic for interaction with sterols. The fact that addition of filipin to PC-cholesterol vesicles causes a complete reappearance of the phase transition characteristic of pure PC is in agreement with such a competition [1]. A stoichiometry of 1 : 1 for filipin-cholesterol complexes is generally assumed [14].

Until now, only a few reports have dealt with the interaction of filipin with plant sterols. Whereas animal and fungal cells mainly contain one major sterol–cholesterol and ergosterol, respectively – in most higher plants, sterols are present as a complex mixture of Δ^5-sterols. Sitosterol, stigmasterol and campesterol are usually cited as the typical plant sterols in tracheophytes [15] (Fig. 1). These sterols are concentrated mainly in the plasma membrane [16]. Plant sterols differ from cholesterol by the presence of an additional bulky alkyl group at C-24 of the side-chain. The high content of polyunsaturated fatty acids in plant membranes, which results in a lower packing of phospholipids compared to that of phospholipids in animal membranes, is probably an important feature for a good fit between plant phospholipids and plant sterols. As in membranes from animal [4–9] and fungal [17] cells, filipin was shown to induce lesions in higher plant membranes [18,19].

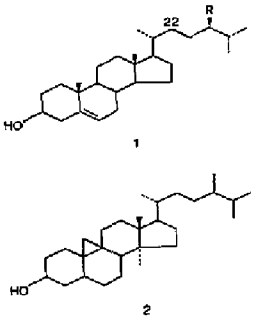

Fig. 1. Structures of plant sterols used in this study. (1) Δ^5-Sterols: R, CH_3: campesterol; R, C_2H_5: sitosterol; R, C_2H_5 and Δ^{22}: stigmasterol. (2) 24-Methylpollinastanol.

Using sterol biosynthesis inhibitors or antifungal chemicals, we have shown that it is possible to obtain plant cell suspensions or whole plants with a completely modified sterol profile [20,21]. Thus, when maize caryopses are grown in the presence of N-substituted morpholines, tridemorph or fenpropimorph, two systemic fungicides, the Δ^5-sterols almost disappear and are replaced by 9β,19-cyclopropylsterols, mainly cycloeucalenol and 24-methylpollinastanol (Fig. 1) [21]. It has been demonstrated that these unusual sterols are incorporated into membranes [22]. Such membranes indeed constitute most suitable material for studying structural and functional roles of sterols in higher plant cells which are still largely unknown.

In the present report, we systematically compared the interaction of filipin with purified membrane fractions from maize roots of varying sterol compositions and that with LUV prepared from soybean PC and different plant sterols using ultraviolet absorption and CD spectroscopy. CD has proved to be the most convenient method for monitoring conformational changes and self-association of polyene antibiotics [2,23].

Material and Methods

Materials. Filipin was purchased from Sigma and given to be approx. 40% pure. In earlier

studies using filipin as a probe for membranous cholesterol, this antibiotic was generally used in its crude unfractionated form. Therefore, with the object of comparing our investigations to the previous ones, we proceeded in the same way. Filipin concentrations were calculated on the basis of ultraviolet absorption ($\epsilon_{355} = 8.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ in methanol). They were also expressed as filipin/sterol molar ratios (r_{st}). Stock solutions (0.01 M) of filipin in DMSO were prepared just before use. Filipin was introduced either as a DMSO solution or as a suspension in buffer, but the final concentration of DMSO in assays never exceeded 1% (v/v). Soybean α-L-phosphatidylcholine (PC) and egg α-L-phosphatidic acid (PA) were obtained from Sigma and used without further purification. Stigmasterol and sitosterol were from Fluka and campesterol from Research Plus. 24-Methylpollinastanol was extracted from maize roots treated with fenpropimorph according to Bladocha and Benveniste [21] as a mixture of (24*R*)- and (24*S*)-epimers [24] and was found to contain 2% 24-ethylpollinastanol. The purity of sterols from commercial sources was checked by gas chromatography and shown to be higher than 93%. Fenpropimorph was kindly supplied by BASF (F.R.G.).

Preparation of vesicles. LUV were prepared in 0.1 M Na_2SO_4 buffered with 2 mM Na_2HPO_4 (pH adjusted to 7.6) by the reverse-phase evaporation procedure of Szoka and Papahadjopoulos [25] from soybean PC, egg PA and sterol(s) in different molar ratios. Soybean PC was selected because it is a major phospholipid in plant membranes [26]. Egg PA was present in all the assays at 10 mol%.

Sterol-free LUV were also prepared. For some experiments, filipin was incorporated into sterol-free LUV by adding 1 ml of 3 mM buffered filipin to 20 μmol phospholipids (PL). The non-incorporated aggregates of filipin were removed by filtration through a Sephadex G-25 column. The composition of such LUV was found to be soybean PC/egg PA/filipin, 80:10:10. The amount of filipin in these LUV strongly decreased after 12 h at 4°C.

Lipid phosphorus was determined as reported in Ref. 27 and sterol content using the enzymatic test 'C system' provided from Boehringer.

Isolation of membrane fractions from maize roots. Maize (*Zea mays*, INRA cv LG 11) caryopses were grown in moist vermiculite in the dark at 25°C. The vermiculite was daily soaked with 0.5 litre water with or without (*R,S*)-fenpropimorph (20 mg/l). Roots were excised after 7 days and membrane fractions were isolated as described previously [28]. Briefly, roots from control and treated plants were chopped into small pieces, then disrupted using a blender in an appropriately buffered medium (0.5 M mannitol/5 mM EDTA/10 mM 2-mercaptoethanol/0.5% (w/v) bovine serum albumin/0.1 M Tris-HCl (pH 8.0)). Membrane fractions were isolated by differential and isopycnic sucrose density gradient centrifugation. Specific subcellular components (endoplasmic reticulum (ER), plasma membrane and mitochondria) were identified by assays for the following markers [28]: ER, NADH–cytochrome-*c* reductase, insensitive to antimycin A, cinnamic acid 4-hydroxylase, *S*-adenosylmethionine-cycloartenol C-24-methyltransferase and cycloeucalenol–obtusifoliol isomerase; plasma membrane, vanadate-sensitive ATPase (pH 6.5) and UDP-glucose: sterol-β-D-glucosyltransferase; mitochondria, antimycin-A-sensitive NADH–cytochrome-*c* oxidoreductase. A microsomal fraction (a 100 000 × *g* pellet) and also three purified membrane fractions were used: a light fraction (*d* 1.10 g/ml) rich in ER, a heavy one (*d* 1.17 g/ml), mainly constituted with plasma membrane vesicles and a fraction enriched in mitochondria (*d* 1.18 g/ml). Fractions were resuspended in 0.1 M Tris-HCl (pH 8.0) with 1 mM 2-mercaptoethanol.

It should be pointed out that apparent densities of the different membranes were not affected by the fenpropimorph treatment. The phospholipid and sterol compositions of the fractions were determined as described elsewhere [28,29]. Protein concentrations were determined according to Ref. 30.

Spectroscopic measurements. CD spectra were recorded on a Jobin-Yvon Mark IV dichrograph with a wavelength accuracy of ±2 nm; all the spectra were corrected for light scattering by vesicles or membrane fractions. $\Delta\epsilon$ is the differential molar dichroic absorption coefficient ($10^3 \cdot cm^2 \cdot mol^{-1}$). Ultraviolet absorption spectra were taken with a Cary 219 spectrophotometer. Experiments were carried out at 25°C.

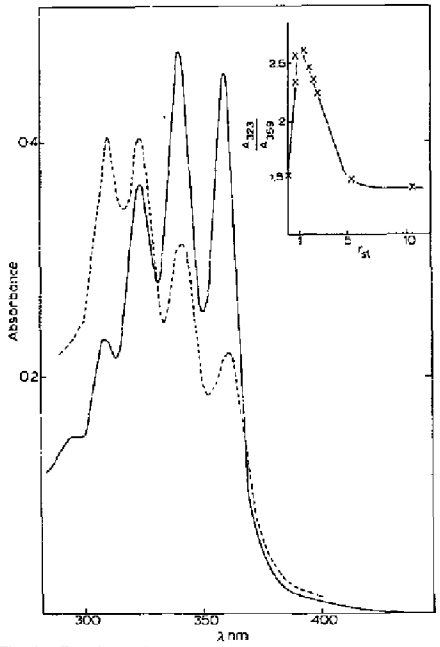

Fig. 2. Ultraviolet absorption spectra of free filipin in buffer and of filipin in the presence of sitosterol-containing vesicles. (———) free $3.5 \cdot 10^{-6}$ M filipin in 0.1 M Na_2SO_4/2 mM Na_2HPO_4 (pH 7.6). (- - - - -) $3.5 \cdot 10^{-6}$ M filipin with soybean PC/egg PA/sitosterol (60:10:30) LUV; PL, $3 \cdot 10^{-5}$ M; r_{st}, 0.3. Inset: A_{323}/A_{359} absorbance peak ratio as a function of r_{st} for soybean PC/egg PA/sitosterol (76:10:14) LUV with filipin concentrations ranging from $0.12 \cdot 10^{-5}$ to $12.5 \cdot 10^{-5}$ M.

Results

Ultraviolet absorption spectra

Filipin aqueous solutions exhibited five absorption bands at 295, 309, 323, 340 and 359 nm, respectively (Fig. 2). This spectrum remained unchanged in the presence of low concentrations (less than $6 \cdot 10^{-5}$ M) of sterol-free soybean PC LUV. In contrast, when filipin was incubated for 30 min with soybean PC LUV containing either individual (stigmasterol, sitosterol or campesterol) or mixed Δ^5-sterols (stigmasterol/sitosterol/campesterol, 55:19:26) in the same range of phospholipid concentrations, a large change in the absorption spectrum of the antibiotic was observed. It consisted of a disappearance of the 295 nm band, a reduced intensity of bands at 340 and 359 nm and an enhanced intensity of bands at 323 and 309 nm as well as a red-shift of the band at 309 nm. Such a change is illustrated in Fig. 2 in the case of sitosterol-containing LUV. The A_{323}/A_{359} absorbance peak ratio was shown to vary as a function of the filipin/sterol molar ratio (r_{st}) (inset, Fig. 2). A maximum was found for a stoichiometry of 1:1. Similar changes in the ultraviolet absorption spectrum of filipin were evidenced in the presence of 24-methylpollinastanol-containing LUV (data not shown).

Circular dichroism spectra

Free filipin in buffer. The CD spectrum of filipin in buffer (0.1 M Na_2SO_4/2 mM Na_2HPO_4 (pH 7.6)) was found to be concentration-dependent (Fig. 3). At low concentrations ($2 \cdot 10^{-5}$ M), the spectrum is characterized by three small negative peaks at 323, 338 and 356 nm ($\Delta\epsilon \simeq -2$) and a positive peak at around 243 nm ($\Delta\epsilon \simeq +2.5$). At filipin concentrations higher than $8 \cdot 10^{-5}$ M, an intense excitonic doublet appears centered at 295 nm with a negative peak at 289 ± 2 nm and a positive one at 305 nm ± 4 nm ($\Delta\epsilon$ 289 $= -34$ and $\Delta\epsilon$ 305 $= +20$ at $16 \cdot 10^{-5}$ M filipin). Such a spectrum will be referred to as the 'type I' spectrum. Both kinds of spectrum are considered to be representative of monomeric and self-associated filipin, respectively [31].

Filipin in the presence of sterol-free vesicles. When filipin was incubated at $2 \cdot 10^{-4}$ M with low amounts of sterol-free soybean PC LUV ($5 \cdot 10^{-6}$ M PL), the 'type I' spectrum, characteristic of the self-associated antibiotic in buffer, was obtained (Fig. 4). In the presence of increasing amounts of vesicles, at first, a decrease of the doublet intensity was observed (the decrease was 40% at about $5 \cdot 10^{-5}$ M PL); then, progressively, the 'type I' doublet became obliterated and a new spectrum was evidenced from 10^{-4} M PL. This new spectrum exhibited a dichroic doublet centered at 298 nm, with a positive component at 292 nm and a negative one at 304 nm. Such a doublet with an opposite sign to that of self-associated filipin in buffer will be referred to as the 'type II' spectrum. It indicates the existence of an interaction between the antibiotic and soybean PC molecules. This

sign inversion of the dichroic doublet probably resulted from a change in the environment of self-associated filipin in a way similar to that reported in the case of zeaxanthine after incorporation into dipalmitoyl PC vesicles [32].

Filipin was also incorporated into sterol-free soybean PC LUV as described in Materials and Methods. CD spectra of such vesicles presented a 'type II' doublet (see Fig. 8). This doublet was shown to disappear after addition of 0.05% (w/v) Triton X-100, indicating that the antibiotic was really incorporated in the lipid bilayer of vesicles.

Filipin in the presence of natural and model membranes containing Δ^5-sterols. The filipin interaction with the various membrane fractions isolated from control maize roots (a 100 000 × g pellet and fractions enriched, respectively, in ER, plasma membrane and mitochondria) was investigated. From analysis of their lipid composition, it was found that all these fractions contained the same sterols (stigmasterol, campesterol and sitosterol) in similar proportions (stigmasterol/sitosterol/campesterol, 58 : 16 : 26) and the same classes of PL (mainly PC and phosphatidylethanolamine); nevertheless, they presented very different sterol and PL concentrations expressed on a protein basis. As shown in Table IA, the plasma membrane-rich fraction appears to be the richest one in free Δ^5-sterols (130 nmol/mg of protein), with a high sterol/PL molar ratio (0.45). In contrast, the ER-rich fraction has a higher PL content but half the sterol content compared to the plasma membranes. Mitochondria have both a low sterol and PL content.

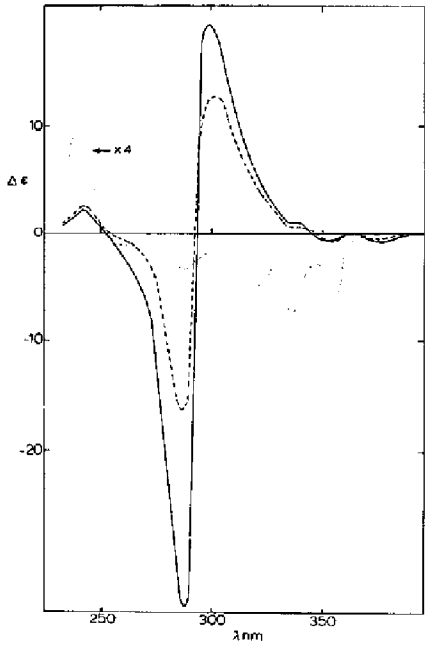

Fig. 3. CD spectra of free filipin in buffer: concentration dependence. (·····) 2.10^{-5} M (×4 amplitude); (------) 12.10^{-5} M; (———) $16\cdot10^{-5}$ M.

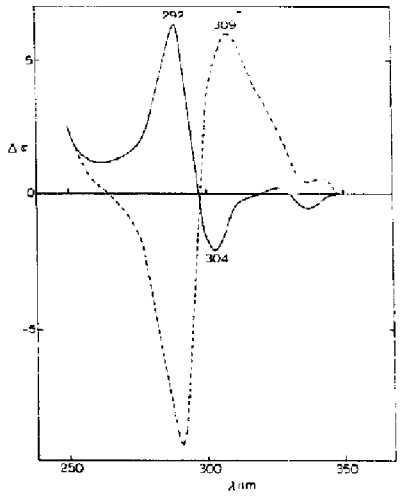

Fig. 4. CD spectra of $2\cdot10^{-4}$ M filipin in the presence of sterol-free LUV (soybean PC/egg PA, 90 : 10) as a function of PL concentration.(------) $0.5\cdot10^{-5}$ M; (·····) $5.4\cdot10^{-5}$ M; (———) $11\cdot10^{-5}$ M.

All of these fractions were incubated with filipin for about 15 min. Because of the interference with protein, spectra were not recorded below 290 nm. Whatever the membrane fraction, similar qualitative changes in the filipin spectrum were evidenced by an enhancement and a red-shift of the three negative peaks, giving maxima located at 334 ± 2, 349 ± 2 and 369 ± 2 nm; moreover, a positive peak was exhibited at 310 nm with a

TABLE I

LIPID COMPOSITION OF MEMBRANE FRACTIONS FROM FENPROPIMORPH-TREATED AND UNTREATED MAIZE ROOTS

Percentage of Δ^5-sterols is shown in parentheses. PM, plasma membrane; Mit, mitochondria.

Membrane fraction	Control (A)			Treated (B)		
	sterols (nmol/mg prot)	PL (μmol/mg prot)	sterols/PL (molar ratio)	sterols (nmol/mg prot)	PL (μmol/mg prot)	sterols/PL (molar ratio)
100 000 × g pellet	90 (94)	0.68	0.13	165 (6)	0.71	0.23
PM	130 (96)	0.29	0.45	185 (8)	0.30	0.62
ER	65 (91)	1.05	0.06	200 (3)	0.94	0.21
Mit	40 (92)	0.32	0.12	60 (5)	0.31	0.19

shoulder at 325 nm. An example is given in Fig. 7 in the case of a 100 000 × g pellet.

The filipin interaction with LUV containing different amounts of either individual or mixed Δ^5-sterol (stigmasterol/sitosterol and/or campesterol) was also investigated. The mixture of Δ^5-sterol (stigmasterol/sitosterol/campesterol, 55 : 19 : 26) which was used was representative of the mean sterol composition of membranes from maize root cells.

At first, let us consider the interaction of filipin with LUV containing individual Δ^5-sterols. Fig. 5 displays, as an example, two aspects of the CD spectra when increasing concentrations of filipin were progressively added to sitosterol containing soybean PC LUV (10^{-4} M PL). At filipin concentrations equal to or less than $4 \cdot 10^{-5}$ M (i.e., for $r_{st} \leq 1.6$), the CD spectra exhibited the following features: (i) the three negative peaks of free filipin were enhanced by a factor of 4–5, depending on the r_{st} values, and red-shifted, with maxima located at 334 ± 2, 349 ± 2 and 369 ± 2 nm; (ii) the positive peak at 243 ± 2 nm was intensified 2–4 fold and another positive peak with a similar differential absorption coefficient ($\Delta\epsilon = +8$ to $+11$) appeared at 249 ± 2 nm; (iii) in the 250–330 nm wavelength region, two positive peaks appeared at 326 and 316 nm. Nevertheless, in this region, the features of CD spectra depended on the structure of the sterol side-chain. Thus, with campesterol and sitosterol, respectively, 24-methyl- and 24-ethylcholesterol, the same peaks, were exhibited; but with stigmasterol, a 24-ethylsterol with an additional double band at C-22, another positive peak was found at 307 nm with a shoulder at 299 nm (Fig. 6). For $r_{st} > 1.6$, the 'type II' doublet appeared (Fig. 5), indicating that a soybean PC–filipin interaction took place. To characterize

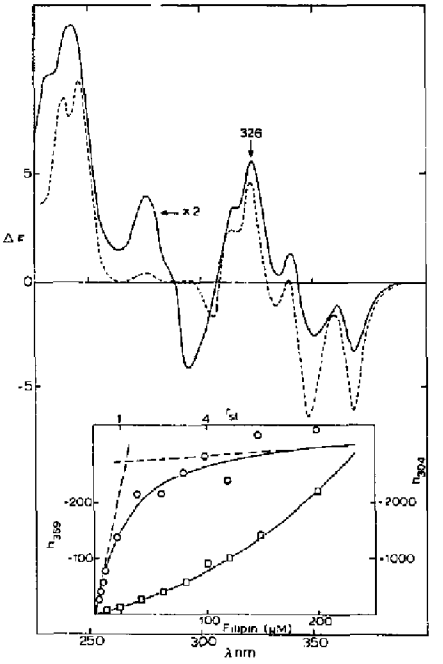

Fig. 5. CD spectra of filipin in the presence of sitosterol-containing LUV (soybean PC/egg PA/sitosterol, 70:10:20) as a function of r_{st}. (- - - - -) 0.2; (———) 6 (×2 amplitude). Inset: intensities of CD peaks at 369 (○———○) and 304 nm (□———□) as a function of r_{st} for sitosterol-containing vesicles. (light path, 1 cm; sensitivity, 10^{-6}).

the general aspects of these spectra with the intent of following the filipin interaction, we chose, on the one hand ($r_{st} < 1.6$), the 359 nm peak intensity, because this peak was very distinct and not present in the free filipin spectrum, and, on the other hand ($r_{st} > 1.6$), the 304 nm negative peak intensity of the 'type II' doublet. Note that the wavelength of this negative peak, first located at 308 nm, was progressively blue-shifted up to 294 nm by increasing filipin concentrations because of the overlapping of the 'type II' doublet by the 'type I' doublet characteristic of free self-associated filipin. The inset of Fig. 5 shows that the intensity of the peak at 304 ± 6 nm was increasing continuously, while that of the peak at 369 nm reached a plateau for an r_{st} extrapolated value near to 1.

Let us now examine the interaction of filipin with LUV containing a mixture of Δ^5-sterols instead of individual sterols. At first, the differential absorption coefficients for an r_{st} value of less than 1 were around twice as high with the mixture of Δ^5-sterols (compare Figs. 6 and 7). Moreover,

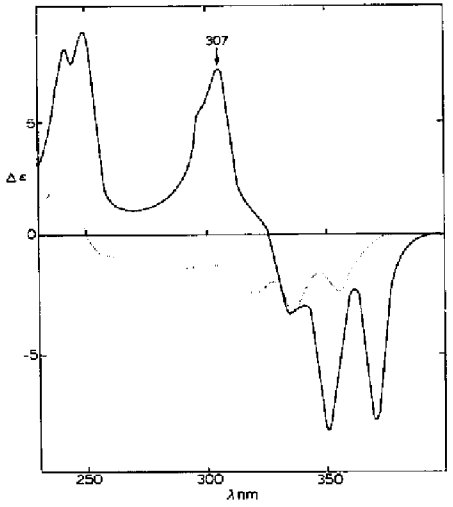

Fig. 6. CD spectra of free filipin in buffer and of filipin in the presence of stigmasterol-containing vesicles. (·····) free $1.2 \cdot 10^{-5}$ M filipin in buffer (spectrum recorded 1 h after dilution); (– ——) $1.2 \cdot 10^{-5}$ M filipin with soybean PC/egg PA/stigmasterol (60:10:30) LUV; r_{st}, 0.9.

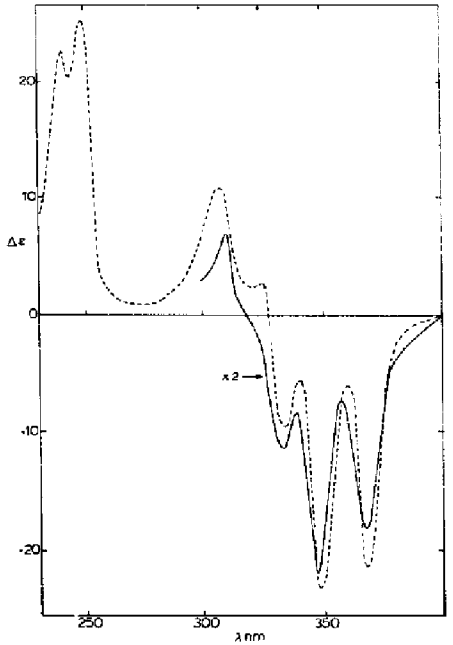

Fig. 7. CD spectra of filipin in the presence of model and maize root cell membranes containing mixed Δ^5-sterols. (– – – – –) $4 \cdot 10^{-6}$ M filipin with soybean PC/egg PA/Δ^5-sterols (70 : 10 · 20) LUV; Δ^5-sterols, stigmasterol/sitosterol/campesterol (55:19:26); r_{st}, 0.4; (———) $8 \cdot 10^{-6}$ M filipin with microsomal membranes (a 100000 × g pellet) from control maize roots; protein, 0.18 mg; sterols, $15.6 \cdot 10^{-6}$ M; PL, $12 \cdot 10^{-5}$ M; r_{st}, 0.5 (×2 amplitude).

qualitatively, the spectrum was quite similar to that obtained with maize root cell membranes, indicating that filipin interaction was not dependent on the protein environment. This spectrum was reminiscent of the spectrum obtained in the presence of stigmasterol, the main sterol of the mixture.

Filipin in the presence of natural and model membranes containing 24-methylpollinastanol. The filipin interaction with different membrane fractions isolated from maize roots treated with fenpropimorph was also investigated. When plant cells or whole plants are grown in the presence of tridemorph or fenpropimorph, two N-substituted

morpholines known to interfere with ergosterol biosynthesis [33,34], the Δ^5-sterols almost disappear and are replaced by 9β,19-cyclopropylsterols [21,35]. The main target in higher plants was shown to be the cycloeucalenol–obtusifoliol isomerase, the enzyme responsible for the opening of the cyclopropane ring of cycloeucalenol to give obtusifoliol [36]. The inhibition of this enzyme by the fungicides induces an accumulation of not only cycloeucalenol but also of other cyclopropyl sterols, such as 24-methylpollinastanol [21,22,35]. These unusual sterols differ from Δ^5-sterols by the presence of a cyclopropane ring at 9β,19 and an additional methyl group at C-14. Moreover, most of them have a C-9 side-chain, whereas major Δ^5-sterols (i.e., stigmasterol) have a C-10 side-chain.

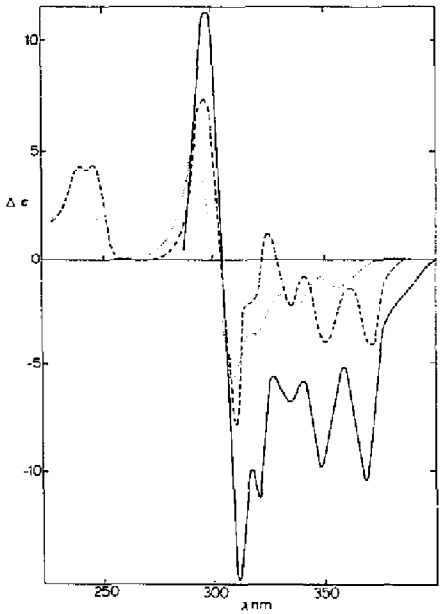

Fig. 8. CD spectra of filipin in the presence of model and maize root cell membranes containing 24-methylpollinastanol and of filipin incorporated into sterol-free vesicles. (------) $0.8 \cdot 10^{-6}$ M filipin with soybean PC/egg PA/24-methylpollinastanol (75:10:15) LUV; r_{st}, 0.15; (———) $8 \cdot 10^{-6}$ M filipin with microsomal membranes (a 100000 × g pellet) from maize roots treated with fenpropimorph; protein, 0.18 mg; sterols, $29.5 \cdot 10^{-6}$ M; PL, $13 \cdot 10^{-5}$ M; r_{st}, 0.3; (· · · · · ·) soybean PC/egg PA/filipin (80:10:10) LUV; PL, $8 \cdot 10^{-5}$ M.

As shown in Table I (part B), 92–97% of total sterols in all the fractions are 9β,19-cyclopropylsterols; 24-methylpollinastanol represents 40, 47 and 40% of total sterols in ER, plasma membrane and mitochondria, respectively. An interesting observation is the presence of the highest sterol concentration in ER instead of plasma membrane. The qualitative and quantitative distribution of phospholipids in the various fractions was shown not to be modified by fenpropimorph.

Whatever the membrane fraction, CD spectra after a 15 min incubation with filipin exhibited the following features (Fig. 8): (i) an enhancement as well as a red-shift of the three negative bands of free filipin with maxima found at 334 ± 2, 349 ± 2 and 369 ± 2 nm; (ii) an intensive 'type II' doublet, slightly red-shifted, with a positive peak at 300 ± 2 nm and a negative peak at 312 ± 2 nm, with a component at 322 nm.

Filipin interaction with LUV containing 24-methylpollinastanol was also investigated. Whatever the value of r_{st}, the 'type II' doublet was observed in addition to negative peaks at 334 ± 2, 349 ± 2 and 369 ± 2 nm (Fig. 8); this observation suggests that, in this case, the filipin–phospholipid interaction took place as soon as small amounts of filipin are introduced.

Discussion

Very few studies dealing with the interaction of filipin with plant sterols have been published [11,37]. Earlier studies devoted to the filipin action were essentially performed with model membranes consisting of multilamellar or small unilamellar vesicles [10,12,37–39] prepared from egg PC and individual sterols. In this paper is presented a systematic study carried out on both biological and model membranes containing exclusively plant sterols and plant lipids using ultraviolet and CD spectroscopy. As biological membranes, we used membrane fractions isolated from maize roots. As model membranes, we used large unilamellar vesicles (LUV) because they afford a far better model of biological membranes than small unilamellar or multilamellar vesicles.

Ultraviolet absorption spectra

We report in the present work a large change in the absorption spectrum of filipin after addition of plant sterol-containing soybean PC LUV. This change consists mainly of an increase in the A_{323}/A_{359} absorbance peak ratio (Fig. 2), in a way similar to that observed in some previous studies [10,37–39]. The same spectral changes were shown to occur whatever the structure of the Δ^5-sterol (stigmasterol, sitosterol or campesterol) incorporated into soybean PC vesicles either individually or mixed with other sterols (stigmasterol/sitosterol/campesterol, 55 : 19 : 26). For all the sterols tested, the A_{323}/A_{359} absorbance peak ratio was found to be dependent on the filipin/sterol molar ratio (r_{st}) and to exhibit a maximum at a value near to 1 i.e., corresponding to the formation of 1 : 1 filipin-sterol complexes (see the inset of Fig. 2). The value of the maximum seems to be related to the structure of the sterol; the better the filipin–sterol interaction, the higher the value of this maximum would be.

We also present evidence for an interaction of filipin with 24-methylpollinastanol-containing soybean PC LUV. In the presence of this 9β,19-cyclopropylsterol, UV spectral changes quite similar to those described for Δ^5-sterols were observed (data not shown).

Finally, we have shown that no significant change in the absorption spectrum of filipin took place after addition of low amounts of sterol-free soybean PC LUV, indicating that the spectral changes which were evidenced above can be mainly attributed to the formation of filipin-sterol complexes. As reported further, the same assumption cannot be held as true in the case of CD measurements.

CD spectra

The modification of the free filipin CD spectrum in the presence of LUV appeared to be a good way to follow the binding of the antibiotic. From the characteristics of the spectra, it was possible to discriminate between different types of interaction and to determine specific features for each sterol structure.

In the presence of sterol-free LUV, a typical spectrum exhibiting the so-called 'type II' doublet was observed only when filipin concentrations larger than $8 \cdot 10^{-5}$ M and PL concentrations higher than 10^{-4} M were used (Fig. 4). A similar spectrum was previously obtained under the same conditions with sterol-free egg yolk PC LUV [31]. Such a spectrum can be interpreted as indicating a filipin–phospholipid interaction. The fact that this interaction can be detected by CD spectroscopy and not by ultraviolet absorption is related to the nature of CD spectra changes consisting of a sign inversion of the dichroic doublet and not of a wavelength shift. Such an interaction between a polyene antibiotic and phospholipids has been reported in the case of etruscomycin (a tetraene) with dipalmitoylPC and egg PC sterol-free vesicles [40] and of amphotericin B (a heptaene) with egg PC incorporated either into small unilamellar vesicles [41], planar membranes [42] or into LUV [43].

When model membranes containing individual Δ^5-sterols were incubated with filipin, the shape of the new spectrum was shown to be dependent on the antibiotic concentration. For an r_{st} value equal to or less than 1, the essential features of the CD spectra were the enhancement as well as the red-shift of the three negative peaks of free monomeric filipin, with maxima located at 334, 349 and 369 nm (Figs. 5 and 6). Similar spectral changes were previously observed with cholesterol-containing vesicles [38,44]. In the 250–330 nm wavelength area of the spectrum, some specific features related to the sterol side-chain structure could be evidenced: whereas in the presence of campesterol (a 24-methylcholesterol) or sitosterol (a 24-ethylcholesterol), two positive bands at 326 and 316 nm were exhibited (Fig. 5), stigmasterol, a 24-ethylcholesterol with an additional double bond at C-22, was shown to induce another positive band at 307 nm, with a shoulder at 299 nm (Fig. 6). These CD spectral features were studied as a function of the filipin concentration. The peak at 369 nm can be retained as characteristic of the filipin spectrum in the presence of sterols. Its intensity was shown to increase and reach a plateau for an r_{st} value near to 1 (inset, Fig. 5), which can be assigned to the formation of filipin-sterol complexes with a 1 : 1 stoichiometry. At higher filipin concentrations (for r_{st} values larger than 1.6), after all the sterol-binding sites are occupied, the antibiotic molecules can interact with phospholi-

pids. The appearance of the 'type II' doublet might be attributed to the formation of complexes between self-associated filipin molecules and phospholipids. Such an interaction is not saturable, as shown by the continuous increase of the negative band at 304 nm (inset of Fig. 5).

In the presence of 24-methylpollinastanol-containing LUV, the filipin–phospholipid interaction was shown to be favoured compared with the filipin–sterol interaction, as evidenced by the appearance of an intense 'type II' doublet as soon as filipin was added (Fig. 8). However, the formation of filipin-sterol complexes was still occurring, since the bands characteristic of such complexes between 330 and 400 nm were observed. The existence of such a competition between 24-methylpollinastanol and phospholipids for interaction with filipin may indicate a lower affinity of the antibiotic for the cyclopropyl sterol in comparison with that of filipin for Δ^5-sterols ((stigmasterol, sitosterol or campesterol). It has been shown that the structural features of sterol for an optimal interaction with filipin were a free 3β-hydroxyl group, a flat tetracyclic nucleus and a side-chain of 8–10 carbon atoms [10,11]. Whereas the first and the third requirements are satisfied, the planeness of the tetracyclic nucleus of the 24-methylpollinastanol can be questioned. 9β,19-cyclopropylsterols were previously believed believed to have a bent conformation [45]. However, recent computer calculations and NMR experiments have shown that if the chair-conformation of the C ring of cycloartenol, a precursor of plant sterols, is changed into a boat-conformation, the molecule acquires a quite stable quasi-planar structure [46]. Thus, the lower affinity of filipin for 24-methylpollinastanol might rather be attributed to the absence of the C-5–C-6 double bond, which was recently reported to be the most effective for optimal sterol–phospholipid interaction [47].

Concerning the comparison of the filipin interaction with model and maize root cell membranes, we report here that the CD spectra of both types of membrane are strongly similar, as evidenced in Fig. 7 by a similar mixture of Δ^5-sterols (stigmasterol/sitosterol/campesterol, 55 : 19 : 26) and a similar r_{st} value (0.4). Moreover, the CD spectra corresponding to the different membrane fractions enriched, respectively, in ER, plasma membrane and mitochondria are shown to be quite identical in spite of significant qualitative and quantitative differences in protein as well as in lipid content (Table I). These results indicate that both the protein component and the presence of several classes of phospholipids in the natural membranes do not interfere with the ability of filipin to interact with Δ^5-sterols. The lack of influence of proteins on the kinetic properties of filipin association with membrane-bound sterols was previously reported in the case of mycoplasma membranes supplemented with exogenous proteins [48]. However, differences in the rate of filipin binding, depending on the accessibility of sterols for the antibiotic, cannot be excluded [38,39]. The situation is different from that of amphotericin B, for which the interaction of the antibiotic with erythrocyte membranes was reported to be influenced by proteins [49].

In conclusion, we have shown that filipin can interact with plant sterols incorporated in both model and natural membranes. The present work indicates that some sterols i.e., the typical Δ^5-sterols (stigmasterol, sitosterol and campesterol) are more suitable than other sterols like 9β,19-cyclopropylsterols to interact with the antibiotic. It is of importance to point out that, under some conditions, filipin can also interact with phospholipids. The interaction of filipin with other plant sterols is under investigation in order to better define the structural features of the sterol molecule for an optimal interaction with the antibiotic.

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