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PROPERTIES OF SPIN LABELLED MEMBRANES OF *FUSARIUM OXYSPORUM* f. sp. *LYCOPERSICI*

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SUMMARY

Growth temperature-induced compositional changes in membranes of *Fusarium oxysporum* provided a test system for study of the relationship between physical properties and composition. Growth at 15 °C was characterized by a decrease in phospholipid content relative to sterol content, a shift in phospholipid composition from phosphatidylcholine to phosphatidylethanolamine and a marked enhancement in the amount of polyunsaturated fatty acids in the phospholipid and triglyceride classes.

Uptake of a spin labelled analog of stearic acid during growth and subsequent solution of the probe in the membranes allowed estimation of viscosity and molecular order of the membranes of live cells and of isolated membrane preparations. Less than 1/20 of the intracellular label was accessible to sodium ascorbate while none was released by sodium dodecyl sulfate. All of the label in live cells was reduced by in vivo respiratory activity above 20 °C but this process could be reversed or avoided by added ferricyanide. A cholestane spin probe was also incorporated into the membranes. The probes were not reduced as readily in isolated membranes and hence fluidity of the membranes could be assessed over a wide temperature range. At low temperatures (−10 °C) a nonlethal, liquid-solid phase transition was indicated in isolated membrane lipids while at higher (lethal) temperatures (40–45 °C), discontinuities appeared in Arrhenius plots of rotational correlation time. Activation energies for isotropic rotation of the stearate probes in the membranes changed markedly in this temperature range and this effect correlated closely with loss of viability of conidial cells. Correlation times for stearate probes showed little variation with growth temperature nor were any breaks in Arrhenius plots of this parameter detected in the range 0–35 °C in whole cells or isolated membranes. The data indicated control of membrane physical properties within close tolerances throughout the physiological temperature range regardless of growth temperature. It was concluded that this homeostatic phenomenon was due to the counteractive effects of sterol/phospholipid ratio, phospholipid composition and fatty acid polyunsaturation since the condensing

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and fluidizing components of the isolated total membranes vary in a reciprocal manner.

INTRODUCTION

The composition of the total membrane phospholipids of late log phase hyphal cells of the fungus *Fusarium oxysporum* f. sp. *lycopersici* was shown previously to vary in a reproducible manner as a function of growth temperature, [1]. The content of polyunsaturated fatty acids increased at suboptimal growth temperatures while a shift in the polar base composition of the major phospholipids occurred which favoured phosphatidylethanolamine over phosphatidylcholine under the same conditions. In view of the temperature dependence of the composition of the phospholipids, the composition of the total membrane lipids has also been examined at different growth temperatures in the present work in order to completely define compositional changes in isolated total membrane preparations.

The physical properties of the membranes of the plant pathogen will depend on induced dipolar bonding interactions in the hydrophobic membrane core as well as on polar interactions of the phospholipids with the aqueous cytoplasmic environment. Hence, the effects of fatty acid and phospholipid composition on membrane physical properties cannot be inferred directly without a knowledge of accompanying changes in sterol composition. Temperature-induced alterations in all these components would be expected to be cooperative in regulating membrane fluidity [2].

An investigation of intact and isolated membrane components and of storage lipids was made to ascertain the effects of composition on physical properties. Lipid-soluble, labelled compounds bearing a stable nitroxide free radical were introduced into the membranes of hyphal cells and microconidia during growth or during membrane isolation. These environment-sensitive probes reported mobility in the region of the hydrophobic acyl chains in the membranes [3]. With thist echnique it was possible to determine what changes, if any, in fluidity in the intracellular membranes occurred [2] as a result of the temperature-induced compositional changes.

Hyphal cells and spores of *Ascomycetes* contain readily identifiable lipid storage bodies containing triglycerides. The lipids in these bodies would be expected to be physically isolated from membrane lipids in the intact hyphae and conidia. Plasma membranes might be expected to be distinguishable from respiratory membranes (mitochondria) on the basis of the chemical reactivity. Spin labels in contact with plant respiratory membrane components are reduced at physiological temperatures [4]. The internal position of the probes in contact with the respiratory chain may be verified by reoxidation with a membrane permeating reagent. Ascorbate is known to rapidly reduce only spin label molecules in the outer layer of a typical phospholipid bilayer array [5]. This reagent can be used to identify spin-labelled membranes with access to the extra cellular space since it cannot readily penetrate the cell interior.

In a previous study of incorporation of a nitroxyl stearate* into growing *Neurospora* hyphae [3] it was found that some of the spin label was incorporated chemically into phospholipids and neutral lipids. In the case of stearic acid and

* Designation for the *N*-oxyl-4',4'-dimethyloxazolidine derivatives of 12- or 16-ketosteric acid. (Also known as doxyl stearate).

cholestane-derived labels, such incorporation is not necessary in order for the label to report on its molecular environment. The effects of protein and sterol interactions with phospholipids on probe mobility may be ascertained by comparing ESR spectra of spin labels located in live cells, isolated total membranes and liposomes composed of purified phospholipids. Analysis of spin label data obtained from such studies of membranes should include order and time dependent parameters to account for the effects of molecular organization and fluidity on experimentally observed ESR line shapes [6]. Probes (such as 16-nitroxyl stearate) which approach isotropic rotation above 10 °C were used in determining rates of molecular motion (correlation times). The method of data analysis has been shown to influence the results in some cases [6]. For this reason more than one method is desirable. The nitroxide moiety of the amphipathic probes which were introduced into the fungal cellular membranes assumed different positions in the hydrophobic matrix as evidenced by notable differences in the temperature dependence of the ESR spectra.

MATERIALS AND METHODS

Spin labelled analogs of stearic acid and of cholestane (4′, 4-dimethylspiro [5α-cholestane-3, 2′-oxazolidin]-3′-yloxyl) were obtained from Syva Corp., Palo Alto, Calif. Buffers and biochemicals were supplied by Sigma Chemical Corp. All solvents were redistilled Mallincrodt chromatographic grade.

Spin labelled cells

Mycelia of *F. oxysporum* f. sp. *lycopersici* were grown in a New Brunswick rotary shaking bath (250 rev./min) at three temperatures, 15, 25 and 37 °C, on Fries medium [7] supplemented with 7.5 μg/ml of the indicated spin probes. The cultures were harvested in log phase after 18 h of growth. The hyphal cell mass, determined on a dry weight basis, was about 10 % lower than that observed after 18 h in the absence of the spin-labelled compounds [1]. The hyphae were separated from microconidia by vacuum filtration and water-washing on a coarse, sintered glass funnel. Microconidia were centrifuged and washed three times with 0.05 M Tris · HCl buffer, pH 7.5 or with the same buffer containing either 20 % glycerol or 1 mM potassium ferricyanide. Samples of hyphae were washed and resedimented in the same way. Bound spin labels were not removed by these procedures.

Spin-labelled membranes

The mycelial mat was cooled to 2 °C and was suspended as a thick slurry in 50 mM Tris · HCl buffer, pH 7.5 containing 1 mM EDTA. A total membrane preparation was prepared by passing the slurry through a Ribi cell fractionator (Sorvall Corp., Norwalk, Conn.) at 25 000 lb/inch^2. The Ribi valve temperature was maintained at 10 °C or lower. Cell wall material and debris were removed by centrifugation at 400 × *g*. The resulting pellet was washed with the extraction buffer and all of the supernatant fluids were combined. A total membrane fraction was isolated from the suspension as a single pellet by centrifugation at 105 000 × *g* for 60 min. The pellet was suspended in 1.0 ml of the extraction buffer and homogenized thoroughly by rapid agitation on a vortex mixer with a 3 mm glass stirring rod. The preparation contained membrane vesicles in a size range 0.1–0.3 μm as determined by phase

contrast microscopy and electron microscopy. Fixation with glutaraldehyde followed by osmium tetroxide staining and imbedding in Epon resin [8] showed that the majority of the empty vesicles were defined by a membrane while some (10 %) contained densely stained lipid material enclosed in a multiple membrane.

Alternatively, the mycelia labelled with 16-nitroxyl stearate were disrupted at 5 °C with glass beads in the presence of 0.5 M mannitol containing 0.01 M Tris · HCl buffer and 1 mM EDTA [7]. The supernatant fluid from a preliminary centrifugation at 1000 × *g* was pelleted after 90 min at 40 000 × *g*, suspended in the extraction medium and layered on a discontinuous sucrose gradient in a centrifuge tube. The gradient was prepared by the method of Nombela et al. [9] and centrifuged at 80 000 × *g* for 2 h in a Spinco model L ultracentrifuge. After equilibration, four fractions were removed from the centrifuge tubes at the 4 gradient interfaces [9] and designated fraction 1–4. Fractions 1 and 2 were pooled as were fractions 3 and 4. The 2 resulting fractions were diluted with 0.5 M mannitol and pelleted at 105 000 × *g*. These pellets were designated as the light (1 and 2) and heavy (3 and 4) membrane fractions, respectively. The latter have been reported to be enriched in mitochondrial enzymes while the former are enriched in reaggregated plasmalemma and microsomal particles [9].

Cell wall preparation

Light microscopic examination showed that 95 % of the hyphal cells were broken in the Ribi cell fractionator while about 50 % of the cells were broken by the glass bead method. Cell wall material was purified from cells disrupted in the Ribi procedure by a previously reported method [10] employing sodium dodecyl sulfate and water washing. Considerable adhering membrane material was removed from the cell walls during washing but the walls retained about 5 % lipid by weight [10].

Lipid analysis

Membrane preparations were extracted with boiling propanol, the extract was dried in vacuo and the lipids were isolated by the modified Bligh and Dyer procedure [1]. The total lipid was fractionated by preparative thin-layer chromatography on binder-free silica. Bands corresponding to phospholipid, sterol, triglyceride and sterol esters were scraped from the plates and extracted with chloroform/methanol/water mixtures. Phospholipids were separated by column chromatography with a sequential solvent system [11] and concentrated under a stream of N_2. Fatty acids were determined by gas-liquid chromatography after methylation [11]. All lipid analyses were repeated 2 or 3 times in separate experiments at the 3 stated growth temperatures.

Quantities of phospholipids were determined colorimetrically after perchloric acid digestion [12]. Ergosterol was determined in the eluates by a modification of the procedure of Rudel and Morris [13]. The absorbance of the sterol or sterol ester reaction product was determined at 460 nm after 50 min and compared with data obtained with ergosterol standards. Separation and identification of sterols was carried out with 5 % OV-101 (Supelco, Bellefonte, Pa.) on 80-90 mesh Anakron ABS support (Analabs, Hamden, Conn.) [14] by comparison with authentic standards. Confirmation of the identity of these sterols was made by analysis on a Finnigan 3100D mass spectrometer system.

ESR spectroscopy

Samples of membrane vesicles, conidia or hyphal cells were dispersed in 0.05 M Tris buffer, pH 7.2 (with or without 20 % glycerol) at a protein concentration of about 5 mg/ml. This suspension was sealed in a thinwall (0.15 mm) Pyrex capillary (0.8 mm inside diameter). The capillary was thermostated in a Varian temperature controlled dewar in a Varian E-3 spectrometer. ESR spectra were recorded at temperatures between −30 and 60 °C. The temperature was continuously monitored directly above the sample with a thermocouple.

ESR data were analyzed where possible by calculation of correlation times (time for rotation of the label through 1 radian) by two different methods assuming isotropic rotation of the probe [6, 15]. Arrhenius plots of correlation times were used to determine whether activation energies for rate of rotation of the spin labels remained constant over a given temperature range or changed abruptly at certain temperatures indicating a structural transition.

Most data recorded below 0 °C reflected neither sufficiently resolved powder spectra nor rapid enough motional characteristics to allow calculation of either the order parameter [16] or correlation time, separately. Examples of these spectra are in the results section. In these cases, an aid in interpretation is provided by simulated spectra which are based on predetermined order, hyperfine splitting and correlation time parameters. Spectral simulations were made by utilizing the treatment of Polnaszek [17].

RESULTS AND DISCUSSION

Lipid composition of membrane preparations

Table I illustrates variations in phospholipid and sterol content of total membrane preparations isolated from mycelia grown at 3 different temperatures. Phospholipid content was markedly low after 18-h growth at 15 °C relative to total sterol estimated as ergosterol. Ergosterol accounted for 83 % of the total sterol while β-sitosterol and a sterol having the same chromatographic retention time as cholesterol (16 %) were also present in membranes from 25 °C grown cells. The total sterol/phospholipid ratio varied from 1.6 for 15 °C grown cells to 0.2 for 37 °C grown cells.

TABLE I

PHOSPHOLIPID AND STEROL CONTENT OF SPIN LABELLED TOTAL MEMBRANE PREPARATION

Standard deviation in phospholipid analyses was estimated to be ±90 μg from 3 replicate experiments. Standard deviation in sterol analyses was ±20 μg. Figures given represent a mean value of 3 separate experiments.

Growth temperature (°C)	Phospholipid (μg)	Sterol (μg)	Sterol ester (μg)	Total sterol (μg)	Total sterol: total phospholipid (μg)
15	465	610	160	770	1.6
25	3712	1100	190	1290	0.35
37	2300	410	105	515	0.22

TABLE II

TEMPERATURE DEPENDENCE OF PHOSPHOLIPID CONTENT OF MYCELIAL MEMBRANE LIPID AFTER 18 h GROWTH

Data for 25 and 37 °C were previously published [1]. They are included here for comparison with 18 h data obtained at 15 °C. These data provide a complete characterization of the major phospholipid classes which are present in the total membrane preparations. Values expressed in mol %.

Phospholipid*	15 °C	25 °C	37 °C
Phosphatidylserine plus phosphatidylinositol	14.9	8.6	14.6
Phosphatidic acid	6.8	5.9	4.4
Phosphatidylcholine	21.3	36.5	44.4
Phosphatidylethanolamine	44.7	36.8	24.6
Dimethylphosphatidylethanolamine	6.7	2.2	4.1
Phosphatidylglycerol plus diphosphatidylglycerol	5.6	10.0	8.0

* Lysophosphatides accounted for 1–4 %.

The hyphal cells which were harvested after 18 h were not necessarily morphologically equivalent. For example, the 15 °C grown cells were in mid-log phase while the 25 and 37 °C grown cells were in late-log phase [1]. Growth for 18 h at the 3 temperatures induced reproducible differences in phospholipid composition as shown in Table II. Data for 25 and 37 °C grown cells were previously reported [1] and are included here for comparison with the data obtained with mid-log phase, 15 °C grown cells. A major change in phospholipid composition occurred through replacement of much of the phosphatidylethanolamine (which represents 42 % of the total phospholipid at 15 °C) with phosphatidylcholine at 37 °C. Hence, the polar base region composition of cellular membranes will depend largely on the growth temperature.

Table III gives the fatty acid composition of the membrane lipids as a function of growth temperature and also includes data for triglycerides and free fatty acids. Fatty acid polyunsaturation was inversely related to growth temperature. It is notable that while both neutral lipids (triglycerides) and phospholipids from 15 °C grown cells clearly show increases in the content of 18 : 3 at the expense of 18 : 1, the concomitant increase in total polyunsaturation is most noticeable in the membrane phospholipid fraction. The calculated number of double bonds per mol of acyl chains (Δ/mol) increased from 1.20 to 1.66 in phospholipids of 37 and 15 °C grown cells, respectively. More important than total unsaturation was the marked shift from mono- to polyunsaturation indicated by a 5.4-fold decrease in 18 : 1 and a 10.4-fold increase in 18 : 3 after growth at 15 °C.

Overall fatty acid changes were not dependent on growth stage (up to late-log phase). However, individual phospholipids isolated from 25 °C grown cells differed in acyl chain unsaturation. For example, phosphatidylethanolamine fatty acids were somewhat more saturated and were composed of 24 % palmitic, 61 % linoleic and 4.6 % linolenic acids. Phosphatidylcholine contained 8.8 % palmitic, 75 % linoleic and 7 % linolenic acids. The data of Table III represents the average acyl composition of the lipid classes.

TABLE III

LIPID AND FATTY ACID COMPOSITION OF TOTAL MEMBRANE PREPARATION

Growth temperature (°C)	Lipid class*	Lipid composition** (% of total fatty acid)	Fatty acid composition (mol % of total lipid)					
			16 : 0	18 : 0	18 : 1	18 : 2	18 : 3	Δ/mol
15	PL	36	19.9	1.8	7.8	52.9	17.5	1.66
25	PL	36	18.5	1.9	7.1	66.2	6.3	1.58
37	PL	36	19.3	0.6	42.2	36.2	1.6	1.20
15	FFA	14	24.0	2.8	10.0	47.9	15.3	1.52
25	FFA	14	21.4	2.9	9.4	60.8	5.5	1.47
37	FFA	14	20.8	2.6	42.4	32.5	1.6	1.12
15	TG	50	13.8	7.9	27.9	38.6	11.8	1.41
25	TG	50	13.1	7.1	29.9	43.0	6.9	1.37
37	TG	50	16.1	7.9	44.7	28.8	2.5	1.10
15	Total	100	17.1	4.3	16.2	46.8	15.7	1.57
25	Total	100	16.5	3.7	16.1	57.1	6.5	1.50
37	Total	100	17.5	4.1	43.8	32.4	2.1	1.15

* PL, Total phospholipid; FFA, Free fatty acids; TG, Triglycerides.

** Less than 1 % of combined mono and digalactosyl diglycerides were detected.

Labelled whole cells and membrane fractions

Fig. 1 shows ESR spectra of 16-nitroxyl stearate located in combined membrane fractions 3 and 4 (curve A) and in the less dense fractions 1 and 2 (curve B). The signal from the more dense fractions is actually a superposition of 3 spectra, the most intense representing the label located in the hydrophobic region of the membranes. Another smaller signal arising from label molecules having more restricted mobility (and therefore being much broader) can be seen under the left hand vertical arrow. The arrow further to the right above curve A indicates the label which has dissolved in an aqueous region giving a very narrow high field signal due to unrestricted motion. Little of the label having highly restricted motion is present in the less dense fractions (curve B) or in whole hyphal cells (curve C) while both show small amounts of unbound label. Some samples of conidia, in contrast, had small amounts of label in an unidentified restricted environment but none in the free state (curve D). There is little discernible difference between spectra arising from the major portion of this spin probe located in the isolated membrane fractions and the spectra of probe bound in live hyphae or conidia. Little of the probe having highly restricted motion was present in total membrane preparations and hence this preparation was the most favorable for assessing the effect of experimental temperature on probe motion.

Localization of 16-nitroxyl stearate in conidia

All of the probe originally present in the culture medium was taken up or metabolized by the cells since none could be recovered from the medium. Conidia obtained from cultures grown on the label exhibited little nitroxide ESR signal unless incubated for at least 1 min with 1 mM $K_3Fe(CN)_6$ to reoxidize the reduced nitroxide (curves A and B, Fig. 2). Addition of 5 mM sodium ascorbate caused a reduction of about 5 % of the label as determined by double integration of curves B and C taking

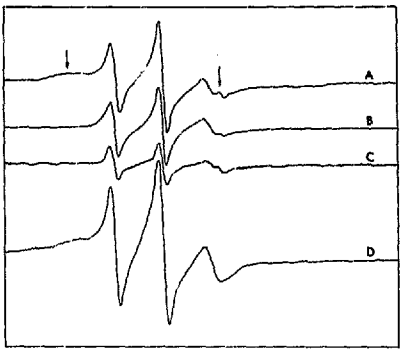

Fig. 1. Spin labelled cells and membrane fractions of *F. oxysporum* f. sp. *lycopersici*. ESR spectra were recorded at 15 °C with samples prepared from 25 °C grown material as detailed in the text. Mol ratios of spin label to lipid fatty acid varied between 1 : 75 and 1 : 150. Spectrometer settings were as listed below. In all figures spectra are centered at 3263 gauss with a field scan range of ±50 gauss (field strength increasing to the right). Vertical arrows above spectrum A represent restricted motion signal (low field side) and a signal from free 16-nitroxyl stearate (high field side).

Spectrum	Sample	Microwave power (mW)	Modulation amplitude gauss	Receiver gain	Time constant (s)	Scan time (min)
A	Mitochondria	12.5	1.0	1.25×10^5	1	16
B	Microsomes	12.5	1.0	5×10^4	1	4
C	Hyphae	20	1.0	8×10^5	3	30
D	Conidia	25	1.0	6.2×10^5	3	30

into account dilution of the conidia by ascorbate solution. This provides an indication of the amount of label located in the membranes which are in contact with the outer aqueous environment since ascrobate cannot rapidly penetrate intact membranes [5]. A combination of $K_2Fe(CN)_6$ and ascorbate reduced 20 % of the bound label.

Microconidia produced on 16-nitroxyl stearate containing medium were normally viable as determined by innoculation into Fries medium. Treatment with 1 % sodium dodecyl sulfate rendered the cells completely inviable. However, only a slight reduction in intensity of the high field ESR signal (curve D) was observed and no relocation of the label occurred due to this treatment. Hence, the detergent, while killing all of the conidia had little effect on membrane structure in so far as the fluidity of the phospholipid matrix is concerned. The detergent-treated cells appeared to be unlysed under phase contrast microscopy. When untreated hyphal or conidial cells were ruptured mechanically, 95 % of the spin label was recovered in the pelleted membrane fractions. Very little label was present in a trigiycerid defraction which floated

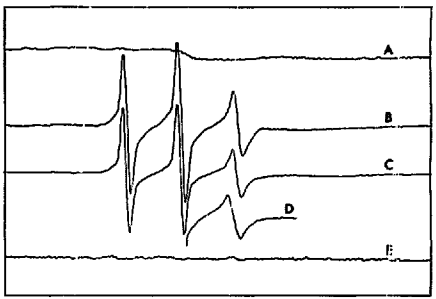

Fig. 2. Sources of ESR spectra of 16-nitroxyl stearate in whole conidia. Whole conidia produced at 25 °C were isolated and washed as explained in text. Spectrum A was obtained before any treatment while Spectrum B was observed after treatment with 0.2 mM potassium ferricyanide. Treatment with excess sodium ascorbate (5 mM) produced Spectrum C. Treatment of conidia with potassium ferricyanide and 1 % sodium dodecyl sulfate did not remove significant label from whole conidia (Spectrum D) but the same treatment eliminated any signal from broken cell wall material (Spectrum E). Spectrometer settings were as listed below.

Spectrum	Sample	Microwave power (mW)	Modulation amplitude (gauss)	Receiver gain	Time constant (s)	Scan Time (min)
A	Conidia	20	0.8	6.2×10^4	3	30
B	Conidia	20	0.8	6.2×10^4	3	30
C	Conidia	20	0.8	6.2×10^4	3	30
D	Conidia	25	0.8	6.2×10^4	1	16
E	Washed cell wall	25	0.8	6.2×10^4	1	16

to the top of the supernatant fluid on centrifugation of membranes at 105 000 $\times$ g.

Although the cell wall fraction isolated from Ribi-disrupted hyphae contained adsorbed membrane material, washing 4 times with sodium dodecyl sulfate and water removed all of the labelled lipid as shown by the lack of ESR signal (curve E, Fig. 2). Since it was not labelled, lipid remaining in the cell wall material [10] was thus not identifiable as adhering membrane fragments but was associated specifically with the cell wall structure.

Effect of the temperature on spectra of membrane bound 16-nitroxyl stearate

Temperature effects on the ESR spectra of 16-nitroxyl stearate labelled total membrane preparations are illustrated in Fig. 3. Relatively little order was evident in the region of the probe above 10 °C as evidenced by the presence of little unsymmetrical broadening of the nitroxide hyperfine ESR spectra. Below 0 °C increasing order and viscosity became apparent. The results suggest a phase transition in the membranes between −20 and −10 °C. Examination of the isolated total phospholipid fraction

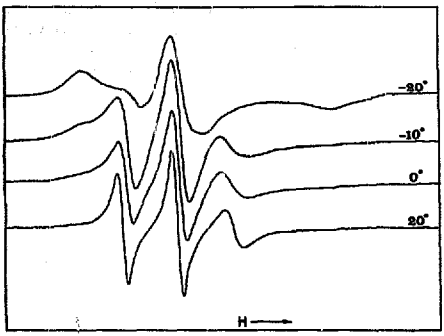

Fig. 3. Effect of temperature on ESR spectra of 16-nitroxyl stearate in total membrane preparation. Membranes containg 16-nitroxyl stearate from 25 °C grown *F. oxysporum* hyphae were dispersed in a minimal amount of 0.05 M Tris buffer, pH 7.5 containg 20 % glycerol. ESR spectra were recorded after 4 min equilibration at the indicated temperatures. Spectrometer settings were: microwave power, 22 mW; modulation amplitude, 0.8 gauss; receiver gain, $5 \cdot 10^4$; recorder time constant, 0.1 s; recorder scan time, 8 min.

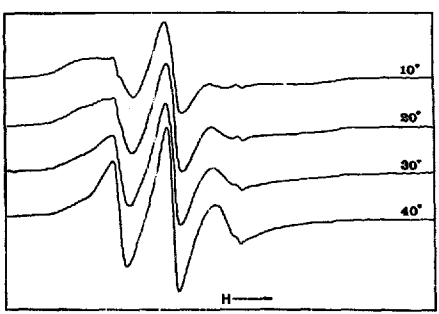

Fig. 4. Effect of temperature on ESR spectra of 12-nitroxyl stearate in total membrane preparation. Total membrane preparation containing 12-nitroxyl stearate from 25 °C grown *F. oxysporum* hyphae was dispersed in a minimal amount of 0.05 M Tris buffer, pH 7.5 containing 20 % glycerol. ESR spectra were recorded after 4 min equilibration at the indicated temperatures. Spectrometer settings were as stated for Fig. 3.

(5 mg) by differential scanning calorimetry also indicated that an exothermic phase transition occurred at −10 °C (Singh, J., personal communication). At −20 °C, the label appears to be present in an environment having properties of a solidified membrane. The suspending medium remained fluid in this experiment due to the presence of 20 % (v/v) of glycerol.

Above 0 °C, line narrowing (Fig. 3) may be attributed to a decrease in the viscosity leading to more rapid rotation of the probe. Above 20 °, the 3 nitroxide hyperfine lines are nearly symmetrical indicating that in the temperature range 20–50 °C, probe motion approximates isotropic rotation.

Effect of temperature on spectra of membrane-bound 12-nitroxyl stearate

Fig. 4 shows spectra obtained with the 12-nitroxyl stearate labelled total membrane preparation. Even at 20 °C there is high viscosity in the hydrophobic regions. The resolution of the low field signals arising from restricted motion of 12-nitroxyl stearate at 20 °C is comparable with that obtained at about −5 °C with 16-nitroxyl stearate or about midway between spectra recorded at 0° and −10 °C in Fig. 3. The data obtained with 12-nitroxyl stearate could not be used to calculate correlation times directly for the *Fusarium* total membrane system.

Effect of temperature on spectra of membrane-bound cholestane-nitroxide probe

ESR spectra of the cholestane probe bound to whole cells showed spin exchange line broadening and hence the label was not distributed in a fluid environment. After disruption of the cells (Ribi method) all of the probe became evenly distributed in the membrane fractions. ESR spectra now exhibited no spin exchange broadening effects. Fig. 5. shows that above 20 °C, a simple 3 line ESR spectrum was obtained from the membranes indicating rotation of the label in a relatively unordered lipid matrix. Below 20 °C, and especially at 0 °C, increasing order and viscosity in the

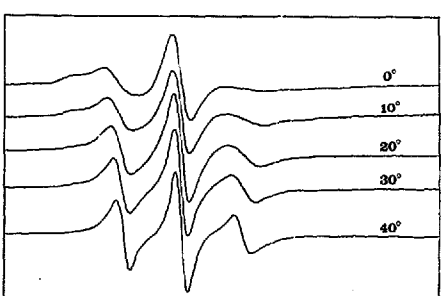

Fig. 5. Effect of temperature on ESR spectra of cholestane probe. Spin labeled total membrane preparation from 25 °C grown *F. oxysporum* hyphae was dispersed in 0.05 M Tris buffer, pH 7.5. ESR spectra were recorded after 4 min equilibration at the indicated temperatures. Spectrometer settings were as stated for Fig. 3.

system was indicated by unsymmetrical broadening of the high and low field resonances. Hence, motion of the probe may depart markedly from isotropic rotation below 20 °C. The region probed by the cholestane label clearly has less fluidity than that probed by 16-nitroxyl stearate.

Comparison of ESR spectra of stearate probes in membranes and isolated lipid-fractions with simulated ESR spectra

Theoretical ESR spectra calculated with the method of Polnaszek [17] are shown in Fig. 6, curves A, B and C. Curve A was obtained for an order parameter (S) of 0.85 ($0 < S < 1$) and a correlation time (τ) of $4 \cdot 10^{-9}$ s. This spectrum is comparable to the spectrum obtained for 16-nitroxyl stearate in the isolated membranes at −20 °C (Fig. 3). The use of larger correlation times and a smaller order parameter also could lead to a simulation of this spectrum. However, the treatment of Polnaszek is not valid for $\tau > 4 \cdot 10^{-9}$ s. Curve B ($S = 0.5, \tau = 4 \cdot 10^{-9}$ s) is similar to that of 12-nitroxyl stearate in the membranes at 10 °C if the small signal due to free label is ignored (Fig. 4). Curve C ($S = 0.25, \tau = 1 \cdot 10^{-9}$ s) approximates the signals from 16-nitroxyl stearate in the membranes at 10 °C.

These comparisons verify that 16-nitroxyl stearate probes a region of relatively low viscosity and little order above 10 °C. Of the available nitroxide stearate probes,

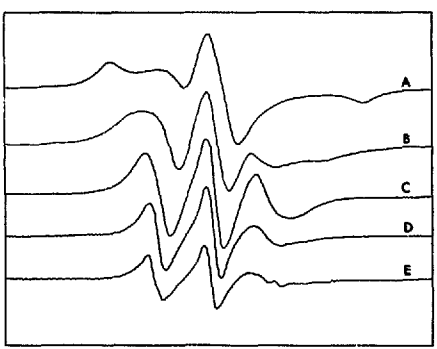

Fig. 6. Simulated ESR spectra and Experimental ESR spectra of 12-nitroxyl stearate in triglyceride and phospholipid preparations. Curves A, B, and C were produced by the method of Polnaszek [17] with the following parameters: Curve A, order parameter (S) = 0.85, correlation time (τ) = $4 \cdot 10^{-9}$ s, hyperfine splitting constants, A_x, A_y = 4.4 gauss, A_z = 32.4 gauss, g_x = 2.0085, g_y = 2.0055, g_z = 2.0027, T_{2A} = 3.0 gauss, T_{2B} = 1.0 gauss; curve B, $S = 0.5$, $\tau = 4 \cdot 10^{-9}$ s, A_x, A_y = 4.4 gauss, A_z = 32.4 gauss, g_x, g_y, g_z, T_{2A}, T_{2B} as in Curve A; curve C, $S = 0.25$, $\tau = 1 \cdot 10^{-9}$ s, A_x, A_y = 4.4 gauss, A_z = 31.8 gauss, g_x = 2.0085, g_y = 2.0055, g_z = 2.0027, T_{2A} = 3.2 gauss, T_{2B} = 1.0 gauss. Spectrum D was obtained with hydrated triglycerides (10 mg) on a quartz plate while Spectrum E was obtained with phospholipid vesicles (5 mg lipid) suspended in 0.05 M Tris buffer, p H 7.5. Spectrometer settings for curve D and E were: microwave power, 20 mW; modulation amplitude, 1 gauss, receiver gain, $2.5 \cdot 10^3$; recorder time constant, 1 s, recorder scan time, 8 min.

membrane bound 16-nitroxyl stearate exhibits the lowest correlation times and least ordering. Hence the motion of this label approximates as closely as possible isotropic rotation for a long chain, amphipathic probe having a fixed location in the hydrophobic region of the membranes.

A triglyceride fraction containing 90 % triglycerides, 8 % phospholipids and 2 % other lipids was isolated by floatation on centrifugation of the total membrane preparation. This material (2 mg) was dissolved in chloroform containing 12-nitroxyl stearate (2 μg) and allowed to dry on a glass plate in vacuo. The plate was then hydrated with water and flushed with dry nitrogen. This procedure produces multibilayers with phospholipid-sterol mixtures [18] but not with neutral glycerides. Curve D, Fig. 6, was obtained at 20 °C for either the $\perp$ or $\parallel$ orientation of the glass plate relative to the spectrometer field indicating the absence of a multibilayer array [6]. It is clear that the labelled triglyceride fraction has even less order and viscosity than that simulated by curve C above ($S = 0.25$). Compared to the 20 °C spectrum of Fig. 4, the spin label in the triglyceride fraction is less restricted in motion than the labelled total membranes at the same temperature. Hence, the presence of triglyceride in the membrane preparations does not lead to overestimation of order or correlation times in the whole membranes. The calculated correlation times for labelled triglycerides are close to 10^{-10} s or considerably less than those of membranes or intact cells.

Curve E, Fig. 6, was obtained with the isolated phospholipid fraction extracted from a total membrane preparation from 25 °C grown hyphae. Liposomes were prepared from the solvent-free lipid fraction which had been previously dried in vacuo by vigorous agitation with 0.05 M Tris buffer containing 12-nitroxyl stearate. Although the spectrum indicates a partition of the label between lipid and aqueous phases similar to that exhibited in the 20 °C spectrum of Fig. 4, the mobility of the label is higher as evidenced by the more symmetrical appearance of the 3 nitroxide resonances. More fluidity is present than in the whole membrane preparation. Approximate parameters may be assigned based on simulated spectra of the type shown in Fig. 6. These values are summarized in Table IV.

Spin labelled phospholipids

Phosphatidylcholine and phosphatidylethanolamine (4–6 mg) isolated from the total membrane preparation from 25 °C grown cells were spin labelled with 4 μg of 16-nitroxyl stearate and sonicated with a 3 mm probe transducer (Branson Corp.) for 1 min in 0.05 M Tris buffer, pH 7.5. Only high field ESR resonances recorded at

TABLE IV

ESTIMATED ORDER PARAMETERS FOR CONIDIA, WHOLE MEMBRANES AND LIPID FRACTIONS CONTAINING 12-NITROXYL STEARATE

Sample	Estimated order (20 °C)	Estimated τ_c (20 °C) ($\times 10^{10}$ s)
Conidia	0.27	25
Whole membrane	0.27	25
Phospholipid liposomes	0.20	5
Triglyceride	0.15	3

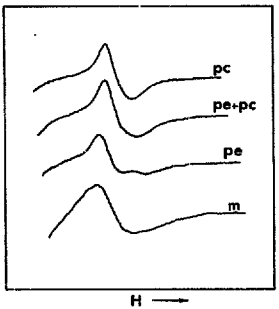

Fig. 7. Spin labelled isolated phospholipids and whole membrane preparation. Isolated phospholipids (5 mg) were spin labelled with 16-nitroxyl stearate at a mol ratio of spin label to fatty acid of 1 : 100. The mixture was sonicated in 0.05 M Tris buffer at pH 7.5 to give liposomes and ESR spectra centered on 3285 gauss were recorded at 10 °C. Only the high field resonance of the spin probe is shown since little difference was observed in the low-field and center-field resonances. Spectrum "m" was obtained under similar conditions with total membrane preparation. Spectrometer settings were: microwave power, 20 mW; modulation amplitude, 0.8 gauss; receives gain, $1.5 \cdot 10^4$; time constant, 0.1 s; recorder scan time, 4 min.

10 °C are shown in Fig. 7 because this portion of the spectrum exhibits the greatest differences in broadening due to motional restriction. Equimolar mixtures of these phospholipids were treated in the same way. Phosphatidylethanolamine in the absence of phosphatidylcholine formed poorly defined large vesicles while in the presence of phosphatidylcholine, liposomes were formed in the size range 5–10 μm as determined by phase contrast microscopy.

While phosphatidylcholine gave a nearly symmetrical resonance, the phosphatidylethanolamine resonance was broadened and partially resolved into 2 signals. The high field signal indicates greater restriction of motion for a portion of the label. Decreasing both modulation amplitude and microwave power did not result in a narrowing or increased resolution of the signals. The phospholipid mixtures exhibited a broadened high field resonance but no resolution of 2 distinct signals. The total membrane signal was even broader than the phosphatidylethanolamine signal but showed no resolution. This experiment indicates that in the mixture as in the membranes there may be a range of liquid environments of varying motional restriction. The membranes show greater broadening than would be predicted on the basis of the approximately equimolar amounts of the main phospholipids which are present in the membrane after growth at 25 °C. This result may be explained by the additional condensing effects of proteins and sterols in the intact membranes.

Arrhenius plots of correlation times for conidia and membranes

Plots of the log of correlation times calculated in 2 ways [6, 15] against reciprocal absolute temperature are shown in Fig. 8 for the 16-nitroxyl stearate probe in

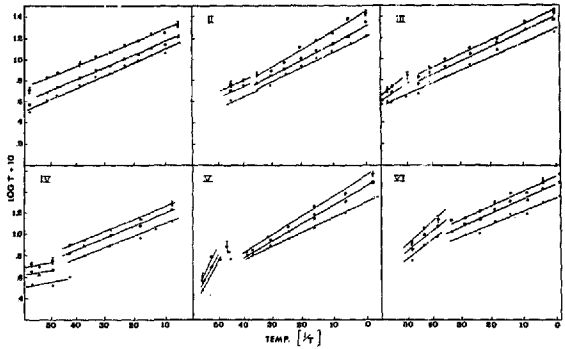

Fig. 8. Arrhenius plots for 16-nitroxyl stearate labelled total membrane and conidia. ESR spectra were recorded at different temperatures as per Fig. 4. Correlation times were calculated in 2 ways; the top most line (●–●) and the bottom line (▲–▲) in each set represents τ as calculated in Cannon et al. [6] while the middle line (■–■) of each set is the correlation time calculated according to Kivelson [15]. Error bars indicate (below 10 °C, near discontinuity, above discontinuity) the precision of several determinations of correlation time. The magnitude of the standard deviation varied with temperature, being greatest in the region of discontinuity and least at lower temperatures.

whole membrane preparations from cells grown at 4 temperatures and for conidia grown at 15 and 37 °C. Correlation times could not be calculated below 0 °C for this label as previously mentioned due to the onset of solidification. In the membranes from 15 and 20 °C grown cells (parts I, II), little change in activation energy for probe motion occurs up to 50 °C. In the membranes from 37 °C grown cells (part VI), there is a decided break in the plots between 35 and 40 °C. A similar break is also suggested by the plots of τ (upper line, calculated according to ref. 6) and τ_C (middle line, calculated according to ref. 15) for 25 °C grown membranes (part III). In contrast, whole conidia grown at all temperatures exhibited pronounced breaks or discontinuities in the Arrhenius plots at around 40 °C. The 25 °C conidia show a discontinuity in this temperature range and an increasing activation energy (part V). Changes in activation energies did not follow a predictable pattern as would be expected for a phase change. Approaching the critical temperature range from higher temperatures also produced discontinuities and apparent changes in activation energy. These observations can be attributed to alterations to essential hydrophobic interactions since the conidia were rendered 100% inviable by heating at 45 °C. Hence, the temperature increase apparently destroyed the essential function but did not induce a simple phase change. ESR spectra gave no indication of departure of probe motion from isotropic rotation in this temperature region.

When the cholestane probe was used to label the isolated membranes no abrupt change in activation energy throughout the temperature range was observed. Hence,

the lethal structural alteration in the membrane can be associated with phospholipid-phospholipid and phospholipid-protein interactions but not with phospholipid-sterol interactions.

CONCLUSIONS

1. Growth temperature-induced compositional changes in hyphal and conidial membranes of *F. oxysporum* provide an opportunity to investigate the homeostatic maintenance of membrane physical properties with spin-labelled lipids. Sterol/phospholipid ratios are markedly enhanced by growth of this organism at low temperatures as has been reported for related fungi. This effect is primarily due to a greater relative decrease in the rate of phospholipid accumulation as compared with sterol accumulation at low temperature.

2. Hyphal and conidial cells of *F. oxysporum* rapidly take up nitroxyl stearate spin labels and retain the probes mainly in membrane components. A cholestane spin label, although bound to cells, did not become evenly distributed in the membranes until cell disruption.

3. Less than 5% of the stearate label in viable conidia was directly accessible to ascorbate reduction. Practically all of the label is assessible to reversible reduction in vivo by reducing systems of the respiratory chain and other membranes. This label may be reoxidized by potassium ferricyanide. Hence, in intact cells little label is associated with isolated storage lipid bodies containing mainly triglycerides.

4. Detergents such as sodium dodecyl sulfate while rendering conidia inviable do not appreciably change the hydrophobic environments of the incorporated spin labels.

5. The 3 spin labels which were employed probe different regions of conidial and isolated membranes. Isolated triglycerides and phospholipids show appreciably more rapid rotation of added 12-nitroxyl stearate than whole membrane preparations or live conidia.

6. Membrane preparations from cells grown at 15, 25 and 37 °C have essentially identical activation energies for probe motion in the range 0–40 °C. Moreover, correlation times at the temperature of growth are very nearly the same ($1.0 \cdot 10^{-9}$ s) for all of the membrane preparations. These results indicate a high degree of control over membrane properties in view of the widely different lipid composition of the membranes of cells from different growth-temperature regimes. It can be postulated that the condensing effects of increasing phosphatidylethanolamine and increasing sterol/phospholipid ratios cancel out the fluidizing effects of the marked enhancement of membrane polyunsaturated fatty acid content [4, 20]. The organism appears to maintain membrane properties within a narrow range with regard to the molecular motion and order experienced by the stearate spin probes. This effect may be due to to complimentary metabolic control over polyunsaturation, methylation of phosphatidylethanolamine and sterol biosynthesis.

7. Above 40 °C, structural alterations occur in the labelled membranes which are most noticeable in whole conidia (grown at all temperatures) and in membrane preparations from cells grown at 37 °C. These nonreversible, disruptive alterations in membrane properties together with temperature effects on proteins and other components render the conidia inviable.

8. The cellular membranes solidify below −10 °C as evidenced by the appearance of a highly viscous and more ordered lipid environment for the 16-nitroxyl stearate spin probe. This phase transition is not lethal or disruptive and is fully reversible on raising the temperature.

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