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THE EFFECT OF PHOSPHATIDYLCHOLINE DEPLETION ON BIOCHEMICAL AND PHYSICAL PROPERTIES OF A *NEUROSPORA CRASSA* MEMBRANE MUTANT

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Summary

By using the choline starvation process it is possible to deplete the membranes of *Neurospora crassa* choline auxotroph *chol-1* of phosphatidylcholine, without affecting the viability of germinated spores or whole mycelium. Spin label probes were used to examine the possible dependence of the physical state of cellular lipids on the presence of phosphatidylcholine in the membranes.

Increased freedom of rotational motion of lipid soluble probes was regularly detected in choline-starved mycelium. The accumulation of neutral lipids (mostly triglycerides) in bulk form was also observed during the choline starvation process. The experiments with isolated and separated lipid classes indicated that the observed increase in fluidity of lipids in choline-starved mycelium is partly due to the difference in physical properties between bulk lipids and membrane lipids. Spin label probe 2N4 (2-propyl-2,5,5-trimethyloxazolidine-*N*-oxyl), which can partition at the membrane-water interface, exhibited easier partitioning among membrane lipids of choline-starved mycelium.

Introduction

In this study, phosphatidylcholine is chosen to be examined with regard to its role in maintaining the physical properties of eucaryotic cell membranes. Phosphatidylcholine is a phospholipid of major interest, because it is often the most abundant phospholipid in animal and plant organelles. It is conspicuously absent, or present only in a small percentage, in bacterial phospholipids [1].

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The specific requirement for phosphatidylcholine has been determined for several membrane-bound enzymes [2]. The importance of phosphatidylcholine for mammals has been indicated in experiments with rats, where deprivation of choline from the diet may cause a decrease in the phosphatidylcholine/phosphatidylethanolamine ratio, accompanied by a large accumulation of neutral lipids and impaired liver functions [3,4]. However, it is easier to examine the role of phosphatidylcholine in some membrane mutants of eucaryotic microorganisms.

The phospholipid composition of cellular membranes can be manipulated by addition of vitamins (choline or insitol) to the growth medium of *Neurospora crassa* membrane mutants: *chol-1*, *chol-2*, and *inos* [5,6]. The *chol-1* strain is blocked in the initial methylation step in the synthesis of phosphatidylcholine from phosphatidylethanolamine because of defective S-adenosyl methionine phosphatidylethanolamine methyl transferase [7]. Suboptimal choline supplementation causes a decreased phosphatidylcholine/phosphatidylethanolamine ratio in the membranes of *chol-1* [6]. The potential usefulness of this mutant for examining the role of phosphatidylcholine in the membranes of eucaryotic cells has not been fully realized. Phosphatidylcholine content can conceivably influence the physical properties of cellular membranes such as viscosity, osmotic sensitivity, permeability or passive transport of solutes across the membrane, surface charge density, the degree of cooperative behavior of membrane lipids, etc.

Spin label probes are commonly used to study the motional characteristics of cellular lipids [8]. Lipid-soluble spin-label probes, when added to the population of living cells, are dissolved among cellular lipids in a very short time. It is possible to design them so that the nitroxide moiety can experience a molecular environment in the vicinity of the polar head groups of phospholipids, or so that it can reach deeper into the bilayer and experience the molecular motion of hydrocarbon tails [9].

Methods

Culture methods

N. crassa choline auxotroph *chol-1a* (isolation number 34486) was obtained from the Fungal Genetic Stock Center (Arcata, Ca.). Stock spore cultures were kept in dry silica gel tubes [10] in a cold room (4°C) and were subsequently used for each new round of large scale production of conidia. Conidia were produced in 125 ml Erlenmeyer flasks containing sporulation medium: 0.5% Bacto-yeast extract/0.5% proteoze peptone (Difco)/2% soluble starch/2% Bacto-agar/4% Bacto-maltose/10 μg/ml choline chloride. Harvesting and washing the spores under sterile condition usually produced about 10^8 spores per flask. The concentration of viable spores was determined by plating on L-sorbose medium [11]. A controlled numer of spores were inoculated into 50 ml of Vogel's minimal medium [12] plus 1.5% sucrose (minimal medium in later text). Choline chloride was added as required. Growth at 25°C proceeded with shaking on a water-bath shaker (Eberbach). For lipid extraction experiments, a known number of spores were inoculated in 3 l of minimal or supplemented medium (10 μg/ml choline). Growth at room temperature (25° C) proceeded with stirring and forced aeration.

Lipid extraction and thin-layer chromatography

Rapid filtration of a 3 l culture generally resulted in 60—80 g of wet mycelia. A representative sample was dried at 80° C for 3 h, while the rest of mycelium was immersed in liquid nitrogen and ground with a mortar and pestle until fine frozen powder was obtained. The cell debris was removed by filtration after over-night extraction in a chloroform/methanol solution (2 : 1, v/v). A Folch wash [13] was performed with 0.25 vol. of 0.88% potassium chloride solution. The lower phase was saved, filtered through anhydrous sodium sulfate crystals and evaporated to dryness with a steam-heated rotatory evaporator. Lipids were collected with several ml of chloroform and separated into neutral lipids and phospholipids on a chromatographic column of silicic acid. Neutral lipids were eluted with diethyl ether, while phospholipids were eluted with methanol. The drying procedure was repeated until each fraction was weighed and dissolved in chloroform.

Neutral lipids were separated by thin-layer chromatography on silica gel G using a pretroleum ether/ether/acetic acid solvent (90 : 10 : 1, by vol.). Sterol specific sprays and lipid standards were used for the identification of spots. Chromic acid-sulfuric acid reagent was used for the quantitative determination of individual neutral lipid classes with cholesterol as a standard [14].

Phospholipids were separated on pre-coated plastic silica gel G thin-layer-chromatography sheets (Brinkmann Instruments, Inc.) by two-dimensional chromatography with chloroform/methanol/water (65 : 25 : 4, by vol.) as the first solvent and chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, by vol.) as the second solvent. Spots were identified with the help of phospholipid standards and specific sprays and were scraped from the thin-layer chromatography sheet for subsequent phosphorus determination [15].

ESR technique and spin labels

Two parameters are extensively used in this study. The partition parameter $f_H = h_{-1H}/(h_{-1H} + h_{-1P})$ is a qualitative indicator of the fraction of spin-label population dissolved in the hydrophobic region of a heterogenous system. The lineheight h_{-1H} is associated with the spin-label population in the hydrocarbon zone, while h_{-1P} is associated with the spin labels in an aqueous phase.

The rotational correlation time τ_c of the spin-label molecule is related to the freedom of molecular motion and can be readily derived from the spectra. If the Lorentzian line shapes and the magnetic field value of 3400 G are assumed,

$$\tau_c = 6.5 \cdot 10^{-10} W_1[(h_1/h_{-1})^{1/2} - 1]$$

where h_1 and h_{-1} are the heights of the low-field and high-field lines of a first-derivative absorption spectrum, while W_1 is the width of the low-field absorption line. Crystal parameters of Griffith, Cornell and McConnell [16] were used to calculate the constant in front of the brackets.

From the slope of the Arrhenius plot for $T\tau_c$ ($T\tau_c$ is proportional to vis-

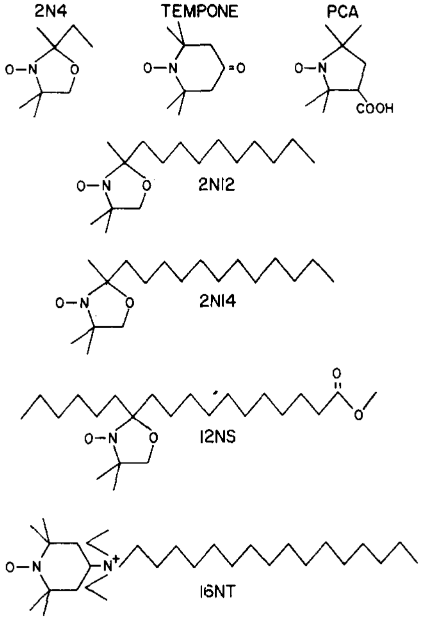


Fig. 1. Spin labels used in this study. Abbreviations are: TEMPONE, 2,2,6,6-tetramethyl piperidone-*N*-oxyl; PCA, 3-carboxyl-2,2,5,5-tetramethyl pyrrolidine-*N*-oxyl; 2N4, 2-propyl-2,5,5-trimethyloxazolidine-*N*-oxyl; 2N12, 2-decyl-2,5,5-trimethyloxazolidine-*N*-oxyl; 2N14, 2-dodecyl-2,5,5-trimethyloxazolidine-*N*-oxyl; 12NS, 12-nitroxide methyl stearate; 16NT, 4-diethylhexadecylamine-2,2,6,6-tetramethyl piperidine-*N*-oxyl.

cosity [17]), the activation energy for viscous flow can also be calculated $E_a = R[d/d(1/T)]\ln(T\tau_c)$ where T and R are the absolute temperature and the gas constant, respectively.

Spin labels 2N4, 2N12 (2-decyl-2,5,5-trimethyloxazolidine-*N*-oxyl), and 2N14 (2-dodecyl-2,5,5-trimethyloxazolidine-*N*-oxyl) (Fig. 1) were synthesized by the same general procedure of Keana et al. [18]. PCA (3-carboxyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl) and TEMPONE (2,2,6,6-tetramethylpiperidone-*N*-oxyl) were synthesized and purified according to the procedure of Rozantsev [19]. Other spin labels such as 12NS (12-nitroxide methyl sterate) and the recently developed surface spin label 16NT (4-diethylhexadecylamine-2,2,6,6-tetramethyl piperidine-*N*-oxyl) were also used. Their synthesis is described elsewhere [20,21].

Temperature runs were recorded on a Varian EPR Spectrometer, Model 4500, equipped with a laboratory-constructed variable temperature control unit accurate to approximately ±0.5°C. In some cases room-temperature spectra were recorded on a Japan Electron Optics JES-ME type spectrometer. All ESR measurements were performed at x-band microwave frequencies.

Procedure for spin labeling of cultures and extracted lipids

Hyphae were collected on filter paper, dried by vacuum suction and divided into 100 mg mycelial pads. Pads were added to tubes with 2 ml of distilled water. In order to prevent spin-label reduction and loss of the ESR signal, mycelia were heat denatured, or oxidants (potassium ferricyanide or *p*-benzoquinone) were added. The heat denaturation process consistend in immersing the tube with mycelium in water at 95°C for 3—5 min. Lipophilic spin labels in ethanol were added in a sufficiently small concentration so that the ratio of mycelial lipids/spin labels was larger than 100. Spin-labeled mycelium was collected by vacuum filtration and placed into a microcapillary ESR tube. Spectra were taken immediately afterwards.

Spin-labeled vesicles were prepared by adding spin label to 40—70 mg of phospholipids in chloroform at a low enough concentration so that the final ratio of phospholipids/spin label was larger than 200. The organic solvent was removed with a stream of nitrogen, and 2 ml of 0.88% potassium chloride solution was added to the tube. The contents of the tube were stirred for 5—10 min and sonicated for 10 min. Vesicles were then diluted with 0.88% potassium chloride solution and filtrated through filter paper (Millipore, 0.22 μm pore size). The resulting vesicle solution was centrifuged at 45 000 $\times g$ for 3 h and the supernatant was disregarded.

Results

Increased fluidity of lipids in choline-starved mycelium

The results with supplemented and starved mycelium treated with *p*-benzoquinone and spin labeled with 2N14, (Table I) may be regarded as most representative of the actual freedom of motion for this spin label in *Neurospora* mycelium. At room temperature, τ_c for 2N14 in choline-starved mycelium is almost 50% lower than in supplemented mycelium.

Heat denaturation did not significantly change the τ_c values for 2N14 in *Neurospora* mycelium (compare Table I). The result of one experiment with supplemented and starved mycelium after heat denaturation is represented in Fig. 2 in the form of Arrhenius plots for τ_c of 2N14. Increased freedom of motion (smaller τ_c) for the spin label in starved mycelium holds for the entire range of physiological temperatures. There is also a change in slope of the Arrhenius plot. The value of E_a is more than doubled in starved culture with respect to supplemented culture.

Both ESR parameters are sensitive to changes in initial choline supplementation and to those in time of incubation. For instance, τ_c for 2N14 can be increased with increasing choline supplementation, while E_a for the same spin label decreased as choline supplementation increased. As a function of incubation time, E_a for 2N14 undergoes changes from 5.5 kcal/mol for spores to 5.2 and 3.9 kcal/mol after 3.5 h and 16 h of incubation in supplemented medium. Incubation in minimal medium resulted in an increase of E_a from its initial values for spores to 9.2 kcal/mol for the stationary-phase starved mycelium.

In order to avoid excessive changes in protein-lipid interaction due to the heat denaturation treatment, oxidant potassium ferricyanide was used in a dual role: (a) to reoxidize the biologically reduced spin labels and (b) to spread the spin-label signal in the polar domain beyond recognition, by the interaction of this paramagnetic molecule (which gives no interfering ESR signal) with the paramagnetic spin label. With high concentrations of potassium ferricyanide (1 M, for instance), only lipophilic spin labels that are not accessible to this

TABLE I

ESR PARAMETERS FOR LIPOPHILIC SPIN LABELS IN *CHOL-1* MYCELIUM

The conditions are described under Methods.

Supplementation µg/ml choline	Spin label	Days of growth	W_1 (G)	$\tau_c \times 10^{10}$ (s)	Temp. (°C)	Treatment
0	2N14	2	1.17	2.87	25	heating
50	2N14	2	1.57	4.37	25	heating
0	2N14	3	1.08	3.06	25	heating
50	2N14	3	1.37	4.58	25	heating
0	2N14	3	1.05	3.10	25	1 M Fe
50	2N14	3	1.03	3.80	25	1 M Fe
0	2N14	3	1.14	2.80	25	heating + 1 M Fe
50	2N14	3	1.20	3.20	25	heating + 1 M Fe
0	2N14	3	1.10	2.65	25	20 mM BQ
50	2N14	3	1.46	4.36	25	20 mM BQ
0	2N12	2	1.06	1.74	35	heating
1	2N12	2	0.93	1.51	35	heating
20	2N12	2	1.07	2.10	35	heating
50	2N12	2	1.31	3.53	35	heating
0	12NS	3	1.46	4.66	55	heating
50	12NS	3	1.90	6.99	55	heating
0	16NT *	3	2.16	8.16	35	heating + 1 M Fe
50	16NT *	3	5.40	33.53	35	heating + 1 M Fe

Fe, potassium ferricyanide; BQ, *p*-benzoquinone.

* In the case of 16 NT, spin label was added while mycelium was still hot after the heat denaturation treatment, and potassium ferricyanide was added when mycelium reached room temperature.

triple charged anion will give a visible ESR signal. For instance, addition of potassium ferricyanide to denaturated supplemented cells, spin labeled with 2N14, resulted in a decrease in spin-label intensity and a decrease in τ_c (Table I). This is consistent with the interpretation that the paramagnetic iron atom preferentially masked the more immobilized fraction of the 2N14 population, which is located in the more polar environment and is therefore accessible to the physical influence of potassium ferricyanide. Potassium ferricyanide alone, without the help of the heat denaturation process, is not so effective in decreasing the τ_c of supplemented cultures. This result may be a consequence of the normal nonaccessability of the internal membranes to the physical action of that charged molecule.

The ESR parameters of several other lipophilic spin labels in *chol-1* mycelium were also measured (Table I). As expected, 2N12 shows little difference in behavior compared with 2N14. The methyl ester of 12-nitroxide stearate, which probes the bilayer interior, seems to be less sensitive to changes in choline supplementation. Surface spin label 16NT is more effective than 2N14 in distinguishing between heat-treated supplemented and starved mycelium, when potassium ferricyanide is also added. The fraction of 16NT population that cannot be reached by the physical action of potassium ferricyanide, even after heat treatment, is practically nonexistent in supplemented mycelium, but definitely exists in starved mycelium.

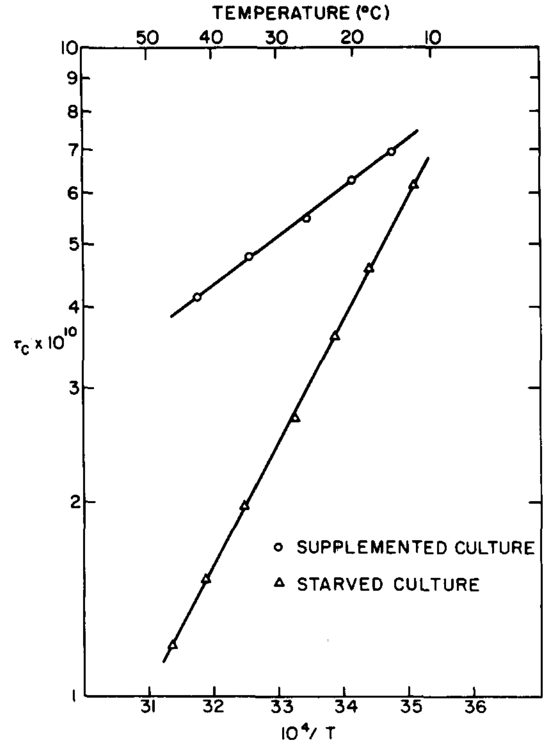


Fig. 2. Temperature run for supplemented (50 μg/ml choline, circles) and starved hyphae (0 μg/ml choline, triangles) labeled with 2N14 after the heat denaturation treatment. Correlation time τ_c is plotted as a function of the reciprocal absolute temperature. Initial inoculation was $2 \cdot 10^6$ spores/ml in minimal medium and $5 \cdot 10^5$ spores/ml in supplemented medium. Growth at room temperature (25°C) proceeded with shaking (140 cycles/min). Both cultures were harvested after 16 h of exponential growth when the absorbance at 560 nm was 0.650. Spin label was added in an approximate amount of $2 \cdot 10^{-9}$ mol/mg (dry weight). The activation energies are 2.9 and 8.2 kcal/mol for supplemented and starved cultures, respectively.

The measuring of τ_c of 2N14 in the whole mycelium is connnected with the difficulties in having the same concentration of spin label in both supplemented and starved mycelium. If the spin label is more concentrated in supplemented mycelium, additional spin label-spin label interaction will lead to additional homogeneous broadening of line widths. This is indeed the case in experiments with 2N14, where the low-field line width is almost always larger for supplemented cells (Table I). Excessive exchange interaction between spin labels may lead to preferential broadening of the mid-field line [22]. However, by subtracting the low-field line width from the high-field line width, for τ_c calculation (Methods) only the inhomogenous broadening process is taken into account, which is predominantly due to the restrictions in molecular motion.

Change in lipid composition and ESR parameters

Lipids isolated from 3-l cultures were separated into neutral lipid and phospholipid fractions (Methods) and determined gravimetrically (Table II). There is a definite increase in size of the neutral lipid fraction of choline-starved cultures.

TABLE II

LIPID SUBCLASSES OF *CHOL-1* MUTANT AND THEIR DEPENDENCE ON CHOLINE SUPPLEMENTATION

See Methods for details.

Supplementation μg/ml choline	Inoculum spores/ml	Days of growth	Mycelium (g dry wt.)	Neutral lipid (mg/g dry wt.)	Phospholipid (mg/g dry wt.)
0	$2 \cdot 10^5$	2	4.6	130	29
10	$1 \cdot 10^4$	2	7.6	27	36
10	$5 \cdot 10^3$	2	8.9	31	41
0	$7 \cdot 10^4$	3	4.2	98	33
0	$7 \cdot 10^4$	3	4.1	95	20
10	$7 \cdot 10^3$	3	11.1	52	32
10	$7 \cdot 10^3$	3	10.4	53	33
10	$7 \cdot 10^3$	3	11.5	55	30

Thin-layer chromatography of the neutral lipid fraction and quantitative determination of lipids (Methods) led to the following conclusion: Triglycerides remain the most abundant lipid species (approximately 50% of the neutral lipid fraction), regardless of choline supplementation. However, according to the results in Table II the absolute amount of triglycerides is considerably higher in choline-starved cultures.

Microscopic examination of the hyphae of choline-starved mycelium disclosed the ubiquitous presence of large sphaerosomes. The lipid specific dye, Sudan Black B, stains preferentially the sphaerosome population. The majority of excess neutral lipids are apparently accumulated in the form of bulk lipids.

Analysis of the phospholipid fraction agreed with the results of Hubbard and Brody [6]. For instance, in one experiment with supplemented culture (10 μg/ml choline) as a standard, starved culture (0.5 μg/ml choline) displayed 51% decrease in its phosphatidylcholine content, 46% increase in its phosphatidylethanolamine content and 83% increase in its phosphatidylinositol content. The lysophosphatidylcholine content also decreased in choline-limited cultures. If choline is completely absent from the growth medium, it is not possible to find a distinctive phosphatidylcholine spot after charring the thin-layer chromatography plate with separated phospholipids. All other spots are clearly visible and in their proper positions.

Separated phospholipids and neutral lipids from cultures grown on different choline supplementation were also used in spin-label experiments (Table III). Triolein clearly represents a good model system for neutral lipids from a starved culture. The activation energy is the same for 2N14 in neutral lipids from a supplemented and a starved culture, but the spin label in neutral lipids from a supplemented culture is slightly more immobilized. Compared with the results for neutral lipids, the values of τ_c (at 40°C) are significantly larger in the ordered phospholipid matrix saturated with water. On the other hand, the activation energy for viscous flow is lower for the spin label in the bilayer matrix.

Partition and permeability studies

Spin label 2N4 (Fig. 1) can partition between the hydrocarbon and water

TABLE III

ESR PARAMETERS FOR 2N14 IN NEUTRAL LIPIDS AND PHOSPHOLIPID VESICLES FROM *CHOL-1* CULTURES GROWN ON DIFFERENT CHOLINE SUPPLEMENTATION

Preparation	$\tau_c \times 10^{10}$ at 20°C	$\tau_c \times 10^{10}$ at 40°C	E_a (kcal/mol)
Neutral lipids from supplemented culture *	6.1	2.3	8.3
Neutral lipids from starved culture *	4.2	1.6	8.3
Triolein *	4.2	1.8	7.2
Phospholipids from supplemented culture **	6.2	4.1	3.2
Phospholipids from starved culture **	7.5	4.5	4.0
L-α-phosphatidylcholine vesicles **	7.7	4.8	3.7

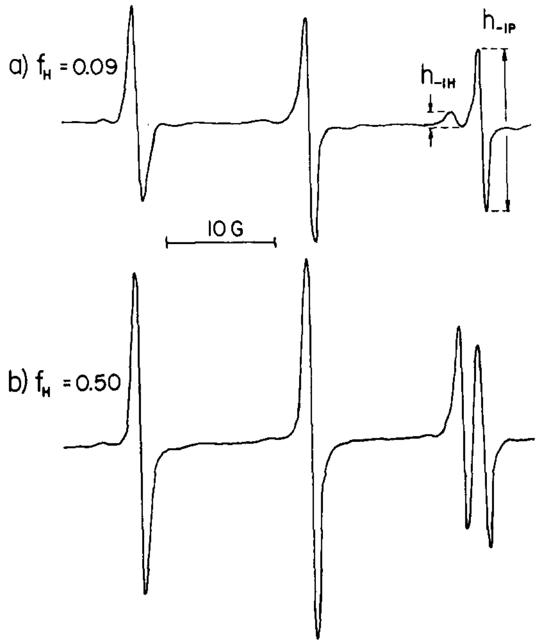
* A small amount of spin label (3 μl of 12 mM 2N14 was added to approximately 50 mg of lipids in organic solvent. The organic solvent was dried with nitrogen flow and a liquid sample of neutral lipids was taken directly for the ESR temperature run.

** Phospholipid vesicles were prepared according to the procedure described in Methods.

phase, giving rise to the two high-field lines due to the different hyperfine coupling constants. The spectra of 2-day-old supplemented and starved mycelium are represented in Fig. 3. The hyperfine coupling constants, measured from Fig. 3, are 16.4 G and 14.3 G, which may be expected for the coupling constant of the oxazolidine ring in the water and hydrocarbon phase, respectively. The hydrocarbon signal has a narrow line width, indicating that there is not much difference in immobilization of 2N4 between the water and hydrocarbon phase. If a high concentration of potassium ferricyanide is applied (1 M), the water signal disappears completely, while the hydrocarbon signal intensity is also reduced more than 100 times. Apparently, the 2N4 signal, characterized by a smaller hyperfine constant, does not originate from the deep regions of the bilayers or internal parts of lipid accumulations, which are inaccessible to the physical action of potassium ferricyanide. Heat denaturation of mycelium increases the partitioning of 2N4 into the hydrocarbon zone.

The hydrocarbon partitioning of 2N4 was absent in *chol-1* macroconidia membranes. The preferential partitioning of 2N4 in starved mycelium is obvious already after the first 24 h of growth and is maintained during the next two days of growth (Fig. 4). The hydrocarbon pool of the 2N4 population in starved mycelium is one order of magnitude larger than that of supplemented mycelium. Obviously, 2N4 can permeate the membrane of choline-starved mycelium much more easily than that of supplemented mycelium. Addition of potassium ferricyanide in a concentration chosen so that the plasma membrane remains intact and that the external polar signal is masked, results in time dependence of ESR parameters for 2N4 (Fig. 5). Since the widths for the $h_{-1\mathrm{H}}$ and $h_{-1\mathrm{P}}$ lines are very similar (0.9 G), the line heights can be used as a good measure for the total spin-label intensity in the hydrocarbon and polar zone, respectively. Obviously, the kinetics of the 2N4 uptake by the hydrocarbon and internal polar regions of the hyphae are dependent on choline supplementation.

Water soluble spin labels can also be used to gain some information about membrane structure and membrane damage. In particular, TEMPONE and PCA (Fig. 1) can be used in the assay of membrane permeability properties. The



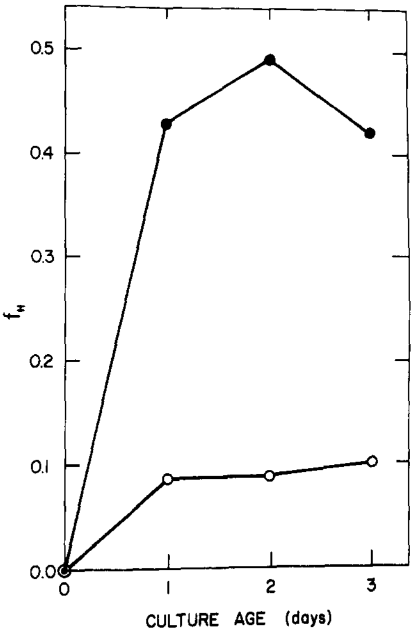


Fig. 3. The partitioning of 2N4 in 2-day-old mycelium (a) supplemented (50 μg/ml choline), or (b) starved of choline (0 μg/ml choline). Initial inoculation was $2.7 \cdot 10^5$ spores/ml. The growth proceeded with shaking, 140 cycles/min, at 30°C. Approximately 150 mg (wet weight) of mycelium were harvested by vacuum filtration and were then transferred to 2 ml of distilled water. *p*-benzoquinone was added to a final concentration of 20 mM to prevent spin-label reduction and 2N4 was added to a final concentration of 0.8 mM. Mycelium was again collected by vacuum filtration and put into a microcapillary ESR tube. Spectra were taken at 30° C. The ratio of high-field line heights (polar/nonpolar) remained the same during the time of measurement. Note the higher value for the partitioning ratio f_H (Methods) in the membranes of choline-starved mycelium (b).

Fig. 4. The partitioning of 2N4 in *chol-1* mycelium as a function of growth in supplemented medium (50 μg/ml choline, open circles), or minimal medium (0 μg/ml choline, filled circles). All samples were prepared as described in the legend of Fig. 3.

method consists of allowing the spin label to equilibrate with the cytoplasmic space. Potassium ferricyanide is also added in a high enough concentration to broaden the spin-label signal in the extracellular space beyond recognition. Therefore, in the ideal case, the spin-label signal will be originating only from the cytoplasmic space, opening the possibility for the measurement of cytoplasmic viscosity [17]. If some cells have a leaky plasma membrane, which is not a good permeability barrier to ferricyanide ions, the spin-label intensity from the cytoplasmic space will decrease proportionally to the percentage of cells with leaky membranes. Membrane lysis is often associated with cellular death.

In the case of *Neurospora chol-1* mycelium, no internal TEMPONE signal is visible when the mycelium is immersed in 1 M potassium ferricyanide solution. A high concentration of ferricyanide can cause the breakdown of the plasma membrane. A ferricyanide concentration which is ten times lower (0.1 M) spreads the low-field line width of the extracellular TEMPONE signal from 0.38 G to 7.70 G, leaving the intracellular signal as the only one that can be

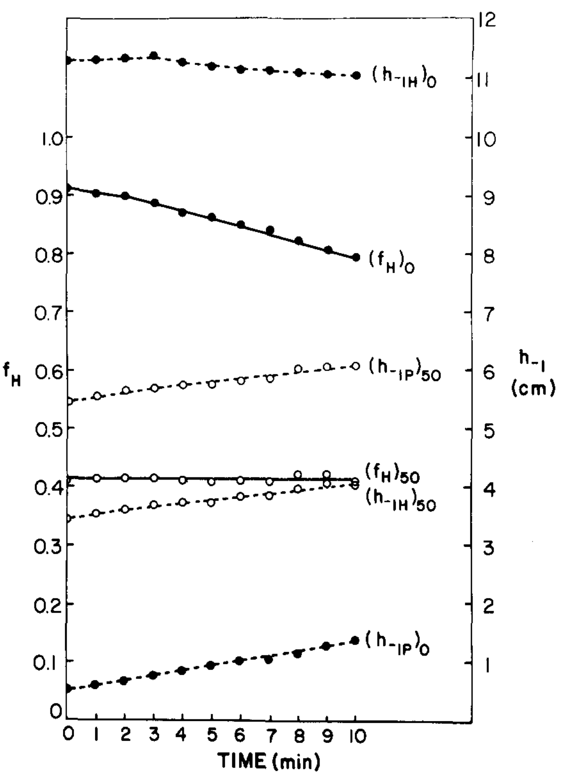


Fig. 5. The time dependence of the partitioning parameter f_H and the high-field line heights h_{-1H} and h_{-1P}. 2-Day-old mycelium, supplemented (50 μg/ml, open circles, index 50) or starved (0 μg/ml, filled circles, index 0) of choline, was incubated in 40 mM *p*-benzoquinone and 0.2 M potassium ferricyanide prior to addition of 2N4. Spin label was added in a final concentration of 0.8 mM. Spectra were taken at time zero corresponding to 10 min after addition of spin label.

seen in the form of sharp three-line spectra. With all other factors kept constant (wet weight of mycelium, *p*-benzoquinone and potassium ferricyanide concentration, ESR instrument setting, etc.), the spin-label intensity from choline-supplemented (50 μg/ml choline) and choline-starved mycelium (0 μg/ml choline) is almost the same. A similar result was obtained with PCA; the plasma membrane of choline-starved hyphae is impermeable to large charged anions, such as ferricyanide, even after four days of the starvation process in minimal medium.

Discussion

For choline-starved *chol-1* cultures, the exponential growth ends after a limited number of mass doublings, and mycelium enters the stationary phase with a drastically changed phospholipid composition and an accumulation of large size lipid-containing sphaerosomes (Juretić, D., Ph.D. Thesis, The Pennsylvania State University 1976). In spite of the change in membrane composition associated with the growth of *chol-1* on choline-free medium, the viability characteristics of this mutant are not changed [23]. By using the ESR method for an estimate of permeability properties of hyphae (TEMPONE and PCA experiments), the same result was obtained for choline-supplemented and choline-starved mycelium: *Neurospora* membranes are impermeable to potassium ferricyanide ions (0.1 M). This is taken as an indication that the growth on choline-free medium does not lead to hyphae lysis and choline-less death of mycelium. Moreover, addition of choline or phosphatidylcholine completely reverses the morphological and other effects of choline starvation. Unlike the inositol-less death of an *inos* mutant depleted of phosphatidylinositol by inositol starvation [24], choline starvation is a nonlethal method for depleting the *Neurospora* membranes of phosphatidylcholine. However, this method is somewhat leacking in specificity. Alongside with phosphatidylcholine depletion, the increase in phosphatidylethanolamine and that in phosphatidylinositol also occur as a primary response to choline starvation. Secondary effects of choline starvation can be considered as consequences of the change in the phosphatidylethanolamine/phosphatidylcholine ratio and/or the phosphatidylinositol/phosphatidylcholine ratio, rather than that of phosphatidylcholine depletion only.

It is well known that some membrane enzymes require a specific phospholipid mixture for their optimal activity [25]. In particular, altered phospholipid composition may have inhibited some membrane-bound enzymes involved in the neutral lipid degradation process, eventually leading to the neutral lipid accumulation in the bulk form. Mitochondrial GTP-dependent Acyl Co A synthetase involved in the activation process of fatty acids is a good candidate because it has a strict requirement for phosphatidylcholine [26]. The lack of an effective regulation mechanism controlling the rate of neutral lipid and phospholipid synthesis would also leave the precursors of phospholipids to accumulate in the storage bodies even if phospholipid synthesis decreased to only a small percentage of the original rate. Triacylglycerols can be a convenient storage form of precursors for the de novo synthesis of phospholipids [27].

One can compare the change in biochemical and ESR parameters associated with the choline starvation process in *chol-1* mycelium. The choline supplementation range from 1 to 50 μg/ml is known to be critical for the growth response and respiration characteristics of the *chol-1* mutant [28]. The change in ESR parameters was a sensitive function of choline supplementation in the same supplementation range. In neutral lipids isolated from both supplemented and starved cultures, the E_a values are close to the values characteristic of the starved mycelium which accumulates an excess of neutral lipids. On the other hand, the E_a values for 2N14 in artificial membranes formed from phospholipids isolated from supplemented and starved cultures, are close to the values characteristic of supplemented mycelium which contains a larger number of phospholipids than of neutral lipids during the exponential growth and in the early stationary phase. It is likely that the distribution of lipophilic spin labels among *Neurospora* membranes and bulk lipids undergoes a change in favor of bulk lipids when bulk lipids are accumulated during the choline-starvation process, so that the ESR parameters for 2N14 in starved mycelium are close to the values characteristic of the same spin label in pure neutral lipids. Experiments with 2N14 labeling of Nematodes [29] having large amounts of neutral lipids (up to 30% of dry body weight) have also indicated a significant difference in the physical characteristics of bulk lipids and phospholipids associated with the membranes.

It is easy to see that the observed difference in the freedom of molecular motion for 2N14 in cultures supplemented and starved for choline cannot be completely explained by the change in the amount of bulk lipids. In order to examine the possible change in fluidity of native membranes brought about by choline starvation, other spin labels were used that were not easily solubilized by bulk neutral lipids. In this study, spin label 2N4 was highly successful in distinguishing between supplemented and starved cultures. The partitioning parameter for this spin label was always higher for the membranes of choline-starved mycelium. The membrane-water partitioning of some spin labels has been frequently used as a measure for the freedom of motion of membrane lipids [30]. Phospholipid-protein interactions are considered to be responsible for the rigidifying effect observed with spin-label probes in model systems [31]. Alternatively, impaired or decreased phospholipid-protein interactions will tend to allow easier partitioning of spin labels in the membranes.

The time dependence of the spin-label intensity in the presence of potassium ferricyanide can be interpreted as representing the influx of the spin label from the external polar space into the membranes of *chol-1* mycelium and subsequent redistribution between the membranes and the internal polar space. In choline-starved mycelium, only the internal polar-signal intensity increases with time, while the hydrocarbon-signal intensity is nearly constant. The rapid saturation of the hydrocarbon regions of choline-starved mycelium with 2N4, before the measurement was taken (during the first 10 min after addition of spin label), may be responsible for the observed time independence of the hydrocarbon-signal intensity.

While a correlation between the partitioning of spin labels and the fluidity of membrane lipids can often be justified by using many different spin labels for probing the same system, the ESR technique can offer only indirect data with regard to the strength of phospholipid-protein interaction. Direct measurements of passive permeability of *chol-1* membranes should be performed to establish the relationship between the physical characteristics of native membranes, isolated organelles and plasma membrane vesicles, and their phosphatidylcholine content. The question of causal connection between the physical state of *Neurospora* membranes and some complex biological phenomena, such as respiration and differentiation, which are inhibited by the choline starvation process [23,28], also merits further investigation.

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