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Role of Lipids in the *Neurospora crassa* Membrane. I. Influence of Fatty Acid Composition on Membrane Lipid Phase Transitions

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Summary. The relationship between lipid composition and phase transition was investigated by differential scanning calorimetry for intact and membrane phospholipid extracts of wild-type (*w/t*) and the *cel*⁻ (Tw 40) mutant of *Neurospora crassa*. The *cel*⁻ (Tw 40) mutant (grown on minimal, sucrose medium supplemented with Tween 40 at ~34 °C) had approximately twice the saturated fatty acid content of *w/t* organisms grown at ~22 °C. The gel-liquid crystal phase transitions of ergosterol-free extracts derived from *w/t* and *cel*⁻ (Tw 40) occur at −31 and −11 °C, respectively. The heats of transition (ΔH) of these extracts were 1 and 13 cal/g, respectively. The addition of ergosterol (the predominant sterol in *Neurospora*) to the phospholipid extracts decreased the observed heats of transition, but did not alter the transition temperature. Intact *Neurospora*, whether *w/t* or *cel*⁻ (Tw 40) did not manifest similar gel-liquid crystal phase transitions in the differential scanning calorimeter. However, an endothermic peak at approximately 30 °C was observed in intact cells and extracted phospholipids of both *w/t* and *cel*⁻ (Tw 40) organisms. This peak was insensitive to the addition of ergosterol, had a low heat content ($\Delta H \simeq 1$ cal/g), and was reversible.

The relationship between lipid constituents and properties of cell membranes has been studied in a variety of membrane preparations utilizing organisms whose membrane lipid composition can be controlled. These studies have thus far been largely restricted to *Achoeloplasma* and bacteria [21, 6, 13, 20]. Most measurements made in such studies are largely indirectly related to *in vivo* membrane properties. Ideally, one would like to alter the lipid composition of an organism's cell membrane in a controlled manner and be capable of measuring some parameters more directly related to membrane activity such as *in vivo* membrane resistance and membrane potential. The assessment of the effect of altered lipid composition on *in vivo* electrophysiological parameters is possible

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with the breadmold *Neurospora crassa* which is unique in allowing: (1) penetration with microelectrodes, and (2) control of its lipid composition through the use of genetic mutants.

In this series of two papers the relationship between the fatty acid content of *Neurospora* phospholipids and the electrophysiological properties of the cell membrane is examined. This first paper reports studies of the physical properties of *Neurospora crassa* wild-type (*w/t*) and the *cel*$^-$ mutant. The fatty acid composition of the two strains are compared and differential scanning calorimetry (DSC) experiments of intact *Neurospora* as well as their extracted phospholipids are described. The effect of ergosterol, the predominant sterol in *Neurospora*, on the calorimetric properties of *w/t* and *cel*$^-$ (Tw 40) phospholipid extracts is also described.

The second paper (*in preparation*) reports the results of membrane resistance and membrane potential measurements made on *w/t* and *cel*$^-$ *Neurospora*.

Materials and Methods

Growth Conditions and Experimental Rationale

Liquid cultures of wild-type (*w/t*) *Neurospora crassa* (strain RL3-8A) were grown in 125 ml Erlenmeyer flasks at room temperature (22 °C) on Vogel's minimal medium [27] supplemented with 2% (w/v) sucrose. To alter the membrane fatty acid composition from that of the *w/t* organism the *cel*$^-$ mutant (strain FGSC165) was chosen. This strain is unable to synthesize fatty acids [10] and, in consequence, requires an exogenous source of fatty acids for growth [14]. By changing the exogenous supply of fatty acids, the fatty acid content of the cell membrane can be altered. For this initial study the saturated fatty acid content of the membrane was increased by growing *cel*$^-$ organisms on palmitic acid. Cultures were grown at 34 °C on Vogel's minimal medium +2% sucrose. 0.4 ml of 10% (v/v) Tween 40 (obtained from Sigma, St. Louis, Mo.) in 95% ethyl alcohol was added to 100 ml of the growth medium. The *cel*$^-$ mutant grown under these conditions is designated in this report as *cel*$^-$ (Tw 40).

Liquid cultures were found convenient and consequently used for isolation of phospholipids, fatty acid analysis, and DSC of *Neurospora*. Since it proved essential to use Petri plate cultures in the electrophysiological experiments, fatty acid analyses of plate cultures were undertaken to determine if the difference in growth medium (liquid *vs.* solid) resulted in differences in cell fatty acid content. Agar media for both *w/t* and *cel*$^-$ (Tw 40) strains differed from their liquid media counterparts only in the presence of 1.5% agar. Agar plate cultures were grown by layering a sterile, lightly scratched disc of PUD-O cellophane (courtesy of DuPont, Wilmington, Delaware) on top of the solidified media, and placing an inoculum of *Neurospora* on the upper surface of the cellophane. The technique for growing *Neurospora* on agar plates has been described by Slayman [24]. As Table 1 demonstrates, the differences in the mole percent of palmitic acid and total saturated fatty acid extracted from the two types of cultures (liquid *vs.* solid) is approximately 5% and is minor in comparison to the differences in palmitic acid and saturated fatty acid content of *w/t* and *cel*$^-$ (Tw 40) organisms. Thus, the type of culture used to grow *Neurospora*

Table 1. Fatty acid composition of *Neurospora* phospholipids (mole percent)

Fatty acid methyl ester	*w/t* (22 °C) liquid[a]	*w/t* (22 °C) plate[b]	*Cel*⁻ (Tw 40) (34 °C) liquid
16:0	14.0	19.4	39.3
16:1	2.6	0.1	1.7
16:2	1.6	–	–
18:0	1.5	0.7	2.3
18:1	6.1	4.2	10.2
18:2	58.7	48.3	35.0
18:3	15.5	27.3	11.5
% Saturated	15.5	20.1	41.6
% Unsaturated	84.5	79.9	58.4
% 16-C	18.2	19.5	41.0
% 18-C	81.8	80.5	59.0

[a] Cultures grown in 125 ml Erlenmeyer flasks on liquid culture medium.
[b] Plate cultures grown in Petri plates on culture medium solidified with 1.5% agar.

does not appear to alter appreciably the percentage of saturated fatty acids present in the extracted phospholipids, and allows the correlation of DSC results obtained from cells grown in liquid culture with electrophysiological measurements which can only be carried out on organisms growing on solid medium.

Isolation of Phospholipid

Cultures were harvested by filtration after 48 hr growth. Harvested cells were washed with distilled water and freeze-dried for 24 hr. Lipids were extracted from the freeze-dried tissue using the chloroform-methanol extraction procedure detailed by Christie [5]. These extracted lipids were subjected to a "Folch" wash procedure also described by Christie [5]. All lipid extracts were evaporated to dryness under nitrogen. Traces of remaining solvent in the lipid extracts were removed by freeze-drying overnight. The dried, purified lipid samples were dissolved in chloroform and stored frozen under nitrogen.

Neutral and phospholipid fractions were separated on a silicic acid column. Approximately 1 gram of silicic acid was used for each expected 25 mg of phospholipid. The lipid, dissolved in chloroform, was applied to the column. Neutral lipids were eluted with 30 ml chloroform, followed by the elution of phospholipids in 30 ml absolute methanol.

Fatty Acid Analysis

The fatty acids of the phospholipids were converted to their methyl esters via a transesterification procedure. Approximately 1 mg of the extracted phospholipid (as determined from phosphorous content) was placed in a test tube and reacted with 1 ml BF_3 Methanol Reagent (Supelco, Inc., Bellefonte, Pa.) at 80 °C for 10 min. Quartz-distilled water was added and the test tube shaken. Cyclohexane was added to extract the fatty acids. The cyclohexane, fatty acid-containing, upper phase was transferred to a new test tube and evaporated to dryness under nitrogen. The dried fatty-acid methylesters sample was then redissolved in 0.1 ml cyclohexane for injection into a gas chromatograph.

The fatty-acid methylesters were separated on a Hewlett-Packard 5750 Research Chromatograph fitted with a stainless steel column containing 10% DEGS (Diethyleneglycol: succinate) on 80/100 mesh chromosorb WAW (supplied by Supelco, Inc., Bellefonte, Pa.). The column was operated at an oven temperature of 160 °C, injection port temperature of 260 °C, and flame detector temperature of 282 °C. Approximately 5λ (50 μg) of sample were injected into the chromatograph for each run. Fatty acids were identified by comparison of the retention times of their methylesters with standards chromatographed under identical conditions. The weight percentage of each fatty acid present was determined by peak integration.

Differential Scanning Calorimetry

The transition temperatures of the extracted phospholipids were determined using a differential scanning calorimeter (Perkin Elmer DSC-2). Microgram quantities of phospholipid dissolved in chloroform were deposited in DSC sample pans, and evaporated to dryness under nitrogen. In experiments concerning the effects of ergosterol, samples were prepared by mixing the appropriate microgram quantities of phospholipid and ergosterol together in a chloroform solution. The mixture was deposited in sample pans under subdued light, and evaporated to dryness under nitrogen. Open sample pans were freeze-dried overnight to remove all traces of solvent. Water in excess was added to each pan immediately before sealing, and the sealed sample pan run against an empty, sealed pan as standard.

Differential scanning calorimetry of intact *Neurospora* organisms was performed by washing freshly harvested *Neurospora* hyphae with distilled water and packing them by filtration. The dense mat of hyphae thus generated was placed in sample pans. Wet sample weights were taken to insure that a sufficient amount of phospholipid would be present in a given sample so as to yield a detectable DSC peak. Intact *Neurospora* were initially studied in the temperature range of 1–40 °C to prevent destruction of the cells by excessive temperatures. Subsequent to these scans in the biological range, the samples were cooled to −23 °C for additional calorimetric studies.

Polarized Light Microscopy

The possibility of some phospholipid structural change occurring but not being detected because of a small heat of transition was explored with polarized light microscopy. Approximately 6 mg of purified phospholipid was mixed with 100λ of water and deposited in a depression slide. The mixture was sealed in the depression with a coverglass, and the slide placed in a Mettler FP 52 microfurnace equipped with an auxiliary supply of cold nitrogen. The Mettler FP 52 and its electronic control unit, FP 5, was used in conjunction with a polarized light microscope. The microscope ocular was replaced with a photoelectric polarized light analyzer so that analyzer output was recorded on a chart recorder.

Results

A. Comparison of w/t and cel⁻ (Tw 40) Fatty Acid Compositions

The fatty acid content of the phospholipid fractions of *w/t* and *cel⁻* (Tw 40) liquid cultures is shown in Table 1. The *cel⁻* mutant grown with palmitic acid supplementation had roughly double the incorporation of palmitic acid in its extracted phospholipids as the *w/t* cells.

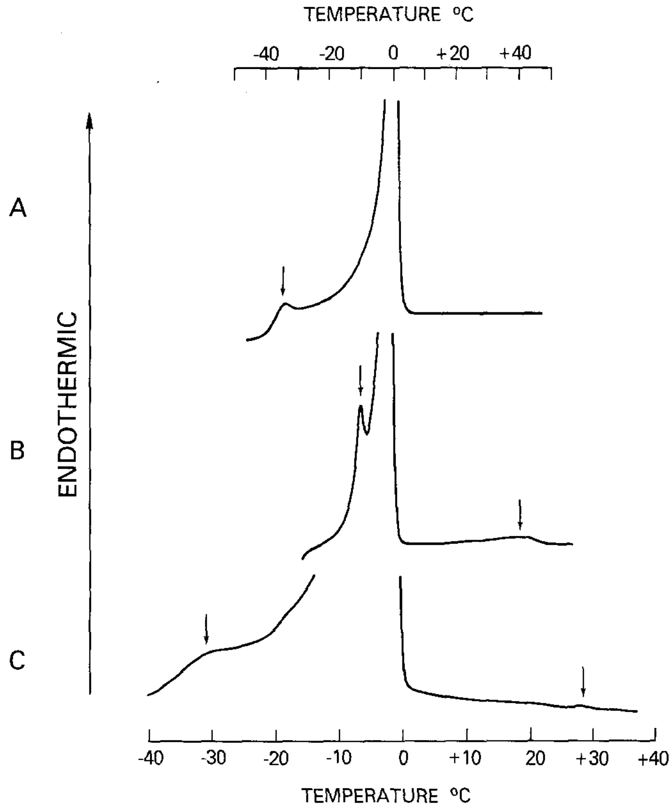


Fig. 1. DSC heating scans of Folch-washed phospholipid extracts of *N. crassa*. (*A*) 3.55 mg *w/t* phospholipid in 2λ water. Calorimeter sensitivity range 5, scanning rate 10°/min. (*B*) 2.5 mg *cel*⁻ (Tw 40) phospholipid in 2λ water. Calorimeter sensitivity range 5, scanning rate 10°/min. (*C*) Same sample as in (*A*) scanned at higher sensitivity to demonstrate presence of high temperature peak. Calorimeter sensitivity range 1, scanning rate 5°/min. Arrows indicate phospholipid phase transitions. Upper temperature scale applies to scans (*A*) and (*B*), lower temperature scale to scan (*C*)

B. Differential Scanning Calorimetry

Intact *w/t* and *cel*⁻ (Tw 40) *Neurospora* and their Folch washed phospholipids were examined by DSC. In preliminary studies phospholipid phase transitions were observed in the absence of an ice-water phase transition by using 50% ethylene glycol. However, since the presence of ethylene glycol can only serve to complicate the phase relationships and has no relevance to *in vivo* phase transitions, the data presented were derived from phospholipid water systems. As shown in Fig. 1, heating scans of Folch washed lipids reveal two large peaks for both *w/t* and *cel*⁻ (Tw 40) organisms. For *w/t* phospholipid in excess water these

Table 2. Heats of transition (cal/g phospholipid): Whole cells and extracted phospholipids

	Wild type		*cel⁻* (Tw 40)	
	High temp. transition	Low temp. transition	High temp. transition	Low temp. transition
Whole cells	1.1 (5)	–	0.7 (5)	–
Extracted PL + 0% ergosterol	0.1 (5)	1.0 (5)	2.5 (5)	13.3 (6)
Extracted PL + 50% ergosterol	0.1 (3)	0.8 (6)	1.3 (8)	6.1 (8)
Extracted PL + 100% ergosterol	–	0.7 (3)	1.0 (7)	2.9 (7)

Latent heat of fusion of water for these experiments was 85.1 cal/g ± 8.9. Values are means. (n) = number of observations.

peaks occur at $\simeq -36$ to -27 °C and at $\simeq 0$ °C. For lipids in excess water extracted from *cel⁻* (Tw 40) organisms these peaks occur at $\simeq -13$ to -10 °C and at $\simeq 0$ °C. The first, i.e., lower temperature, peak in each scan represents the phospholipid crystalline gel-liquid crystal phase transition. The 14–26° shift of this peak to a higher temperature in *cel⁻* (Tw 40) phospholipid samples is attributable to the higher mole percentage of saturated phospholipids in these samples and is in agreement with the findings of other investigators [18, 4]. Table 2 lists approximate values for the heats of transition for the lipid peaks obtained by integration of peak areas. These values are in general agreement with values expected for phospholipid phase transitions [22, 3]. The second, larger peak in these scans represents the melting of ice.

A third, high temperature, endothermic peak is seen in DSC scans of both *w/t* and *cel⁻* (Tw 40) phospholipids. This peak occurs at $\simeq 42$ °C in *cel⁻* (Tw 40) samples and has a heat content (ΔH) of 2.5 cal/g phospholipid (Fig. 1 *B*). In *w/t* samples a similar peak occurs at a lower temperature (29 °C) and because of its smaller heat (0.1 cal/g phospholipid) requires increased calorimeter sensitivity to be seen (Fig. 1 *C*).

Cholesterol has been shown to decrease the observed heat of transition (ΔH) of phospholipid-water systems. This is true of lipid-water systems containing a single phospholipid [16], as well as for systems containing mixed lipids extracted from biological membranes [8]. Since ergosterol is the major sterol in *Neurospora* [9], it was of interest to determine the effect of ergosterol on the phase transitions of *N. crassa* lipid extracts. Referring to Fig. 2 and Table 2, increasing concentrations of ergosterol reduce the heat (ΔH) absorbed during the low temperature phase transition of *w/t* and *cel⁻* (Tw 40) phospholipids. However, a lowering of

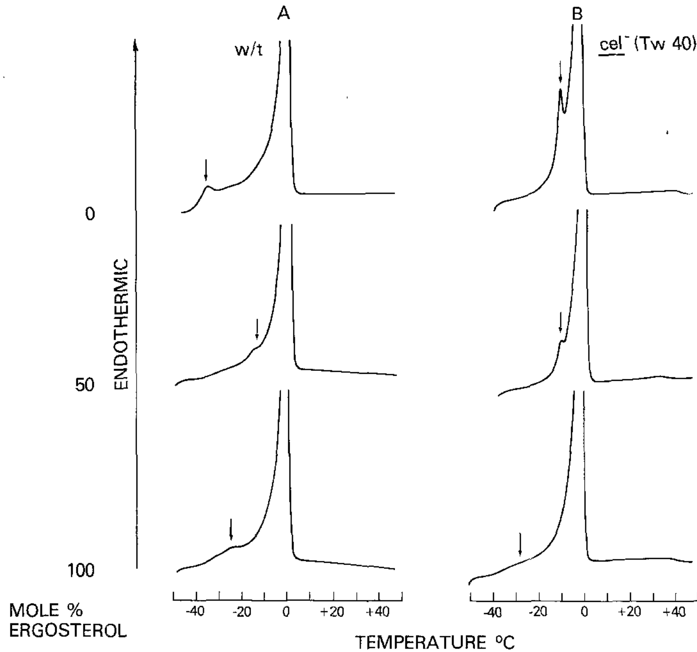


Fig. 2. Effect of ergosterol on gel-liquid crystal phase transitions of Folch-washed *Neurospora* phospholipids. (*A*) Heating scans of *w/t* phospholipid; (*B*) heating scans of *cel*⁻ (Tw 40) phospholipid. Upper traces 0 mole percent, middle traces 50 mole percent, lower traces 100 mole percent ergosterol. All scans recorded at sensitivity range 5, scanning rate 10°/min

Table 3. Temperature of *Neurospora* phospholipid phase transitions[a]

	Wild type		*cel*⁻ (Tw 40)	
	High temp. transition	Low temp. transition	High temp. transition	Low temp. transition
Whole cells	+29(3)[b]	–	+28 to +29(4)	–
Extracted PL+ 0% ergosterol	+28 to +29(3)	−36 to −27(5)	+40 to +43(3)	−13 to −10(6)
Extracted PL+ 50% ergosterol	+29(1)	−33 to −14(5)	+33 to +37(5)	−13 to −9 (7)
Extracted PL+100% ergosterol	–	−29 to −24(3)	+37(4)	−13 to −10(6)

[a] Range of peak values (°C).
[b] (n) = number of observations.

the temperature for this transition as a function of increased ergosterol concentration does not occur (Table 3).

Considering now the high temperature peak as a function of ergosterol, representative DSC scans are shown in Fig. 3. The heat content

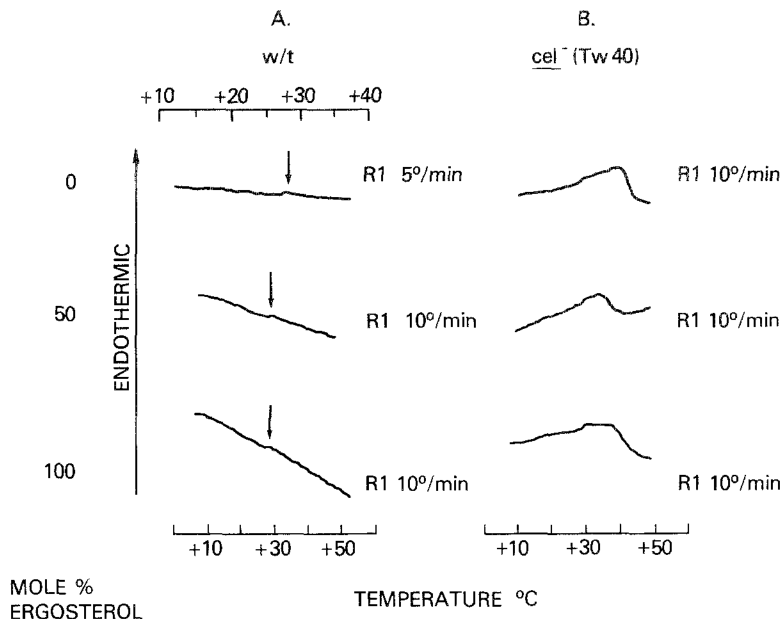


Fig. 3. High temperature peaks of (*A*) *w/t* and (*B*) *cel*$^-$ (Tw 40) Folch washed phospholipid as a function of varying mole percentages of ergosterol. Calorimeter sensitivities (range) indicated as mcal/sec and scanning rates indicated as °/min

of the high temperature peak of *w/t* phospholipid is independent of ergosterol concentration whereas the heat content of the *cel*$^-$ (Tw 40) peak decreases in the presence of ergosterol (Table 2). The temperature at which this peak occurs is independent of the ergosterol concentration (Table 3) for both *w/t* and *cel*$^-$ (Tw 40) samples.

Intact *Neurospora* hyphae were examined in the DSC to determine if it is possible to detect a membrane lipid gel-liquid crystal phase transition in the intact organism. DSC scans of intact *w/t* and *cel*$^-$ (Tw 40) *Neurospora* are compared with scans of extracted phospholipids in Figs. 4 and 5. Hyphae were initially scanned at high sensitivity (range 0.5) in the temperature range of 0 to 37 °C. Such scans yielded one small, reproducible, endothermic peak whose maximum occurs at 28 to 29 °C. Samples were then cooled to −43 °C. Subsequent heating scans then revealed a large, broad-based endothermic event occurring in both *w/t* and *cel*$^-$ (Tw 40) samples at the approximate melting point of ice. This peak remained reproducible after samples had been heated to 47 °C. No evidence of a phospholipid phase transition occurring below the melting point of water was found for either *w/t* or *cel*$^-$ (Tw 40) intact hyphae.

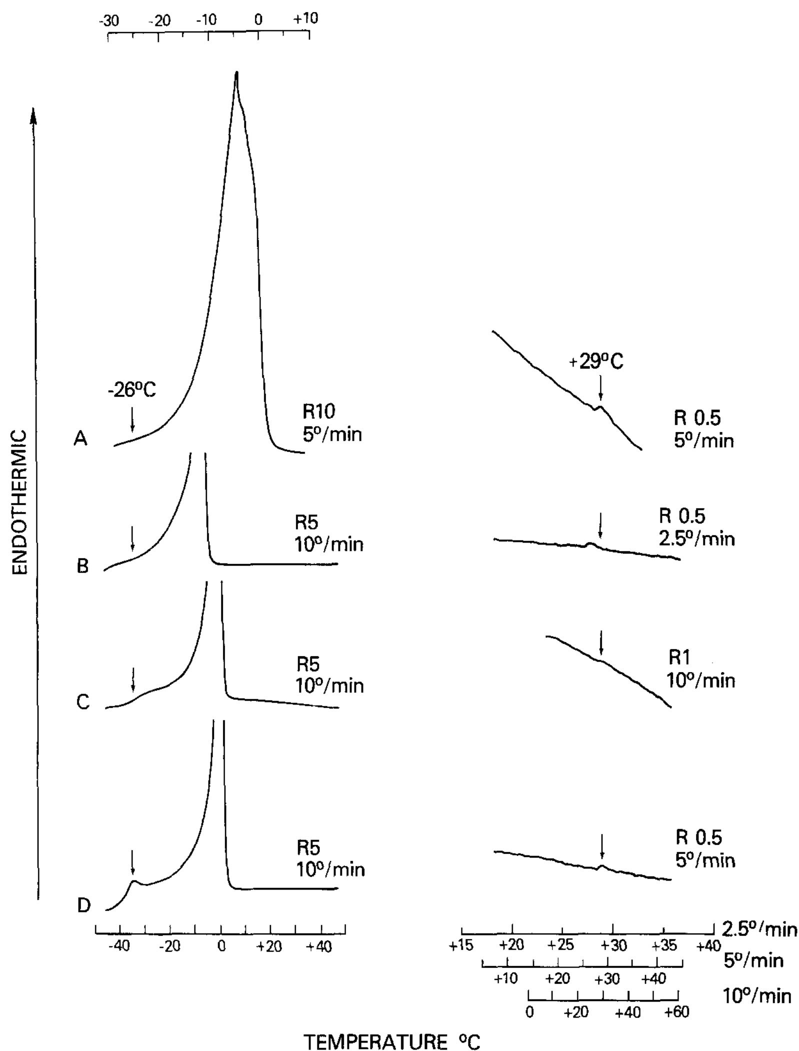


Fig. 4. DSC heating scans of *w/t N. crassa* grown on minimal+sucrose medium. (*A*) whole cells; (*B*) nonwashed phospholipid extract; (*C*) Folch-washed phospholipid extract in the presence of 100 mole percent ergosterol; (*D*) Folch-washed phospholipid. Calorimeter sensitivities (range) indicated as mcal/sec and scanning rates indicated as °/min. Upper left temperature scale applies to scan (*A*). Lower left temperature scale applies to scans (*B*) (*C*) and (*D*)

In an effort to determine the nature of the difference between intact *Neurospora* and Folch-washed phospholipids, DSC scans of nonwashed lipids were run. The nonwashed lipids yielded scans similar to intact hyphae: no evidence of a low temperature, gel-liquid crystal phase transition, and a small endothermic peak whose maximum occurs at ~ +28 °C.

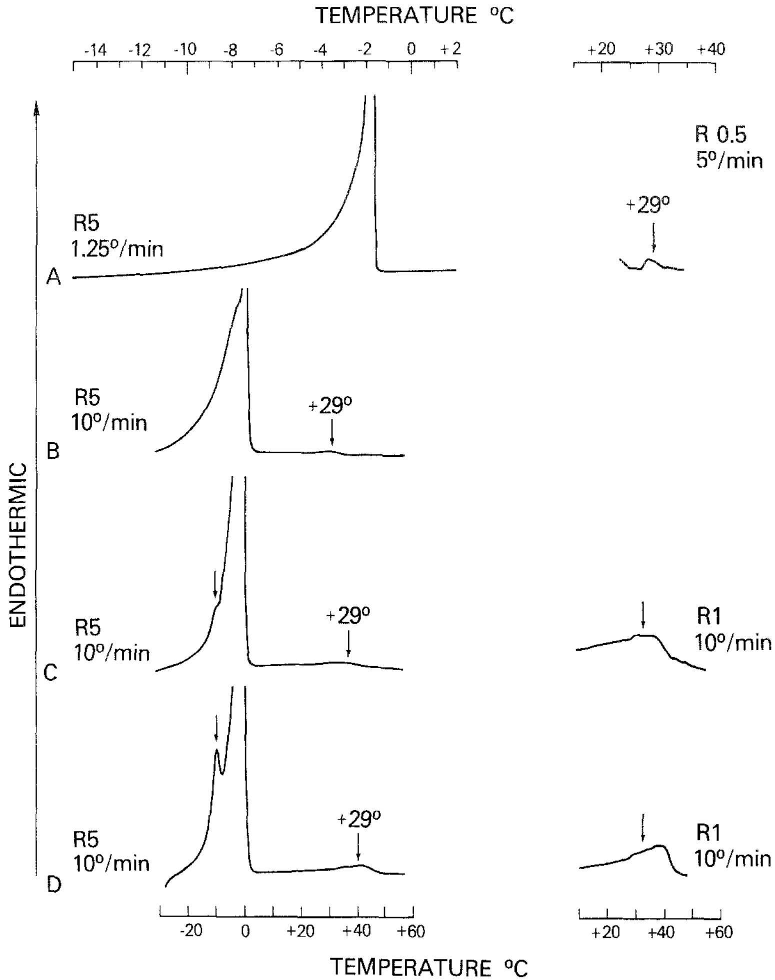


Fig. 5. DSC heating scans of *cel⁻* (Tw 40). (*A*) whole cells; (*B*) nonwashed phospholipid extract; (*C*) Folch washed phospholipid extract in the presence of 100 mole percent ergosterol; (*D*) Folch washed phospholipid. Calorimeter sensitivities (range) indicated as mcal/sec and scanning rates indicated as °/min. Upper temperature scales refer to scans (*A*)

Scans of nonwashed and Folch-washed phospholipids are compared in Fig. 6. In a further effort to determine what is removed from *Neurospora* lipids by the Folch washing procedure, an absorption spectrum was run on the Folch-washed supernatant. The supernatant exhibited a marked increase in absorption with its maximum occurring at 260 mμ indicating the presence of nucleic acids.

C. Polarized Light Studies

The possibility of some phospholipid structural change occurring with an undetected heat of transition was ruled out by polarized light microscopy. *Neurospora* phospholipids in lipid-water mixtures were examined by polarized light microscopy over the temperature range of −30 to +75 °C, and at scanning rates of 1 and 2°/min. Light intensity changes were observed during heating and cooling scans only in the temperature regions of the crystalline gel-liquid crystal phase transitions and nowhere else. Thus, polarized light microscopy experiments support the DSC data in detecting the gel-liquid crystal phase transitions and in finding no other major structural changes.

Discussion

Folch washed phospholipids derived from *w/t* and *cel⁻* (Tw 40) *N. crassa* exhibit gel-liquid crystal phase transitions. These transitions occur at ∼ −31° and at ∼ −11 °C for *w/t* and *cel⁻* (Tw 40) lipids, respectively. The difference in transition temperatures of *w/t* and *cel⁻* (Tw 40) phospholipids is not attributable to differences in phospholipid bases. The phospholipid bases of *w/t* and *cel⁻* (Tw 40) are the same [Stuart Brody, *personal communication*]. The increase in the phase transition temperature (T_c) by ∼20° is attributable to the increase in saturated fatty acid. Increased saturated fatty acid incorporation has been reported under similar conditions [2]. The finding of a change in T_c to a higher value with an increased proportion of saturated fatty acids is consistent with similar observations made by other investigators on synthetic phospholipids [4, 7], biological membranes [18, 11], and derived phospholipids [12].

That the observed low temperature endothermic peaks are indeed gel-liquid crystal phase transitions is supported by a number of points: (a) X-ray diffraction studies[1] of *Neurospora* lipid-water mixtures at 25 °C yielded low angle reflection patterns expected of phospholipid lamellar structures. The wide angle reflection lines were diffuse, indicating that the extracted phospholipids exist in a liquid crystal state at 25° as predicted by the DSC results. (b) The values herein reported for the gel-liquid crystal phase transition of *N. crassa* lipids are consistent with the heats of transition found by other workers for biological membranes and their derived phospholipids. The heats of transition (ΔH) for *w/t* and *cel⁻* (Tw 40) phospholipids are 1 and 13 cal/g, respectively. Other

1 *See* acknowledgments on p. 46.

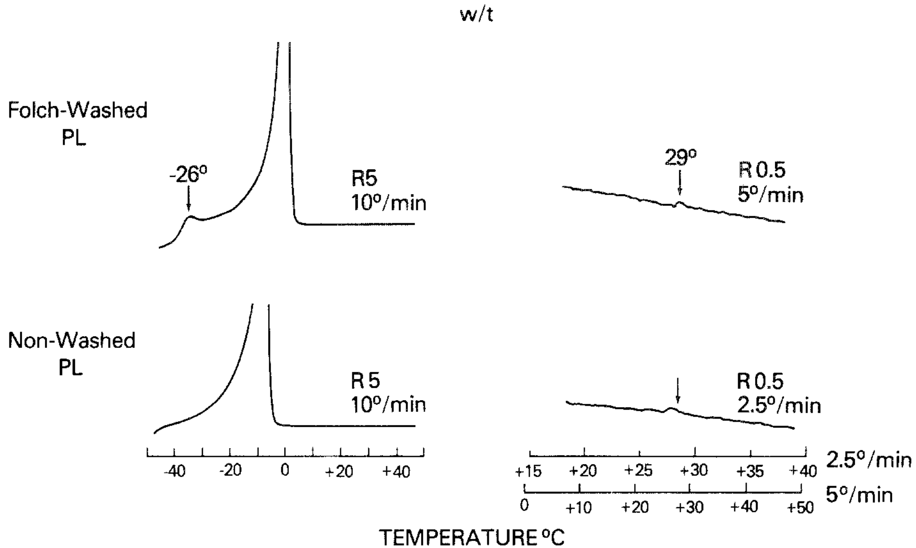
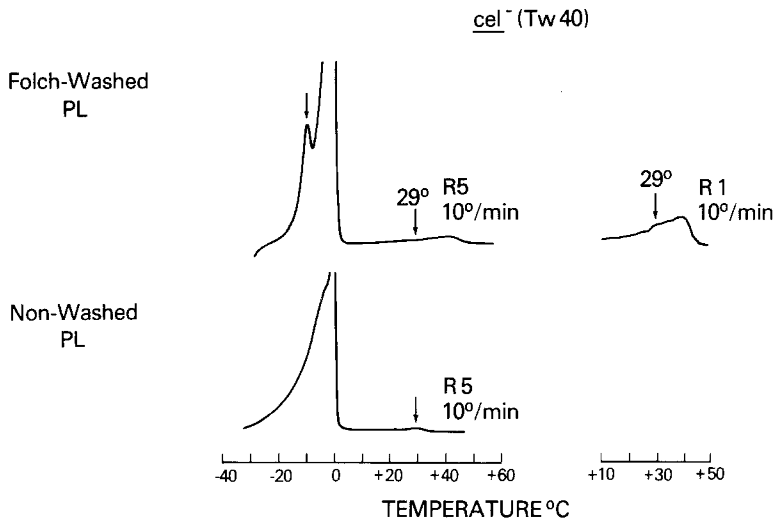


Fig. 6. Comparison of DSC scans of Folch-washed and nonwashed phospholipid extracts. Washed lipids exhibit low-temperature, gel-liquid crystal phase transitions, and endothermic peaks occurring at 29 °C. Nonwashed lipids exhibit 29 °C endothermic peaks but not

workers [22, 3, 19, 26] report values ranging from 2 to 12.6 cal/g. (c) The occurrence of gel-liquid crystal phase transitions at temperatures around 0 °C or below has been reported for other biological systems. Mason and Abrahamson [17] report −28 °C and −31° as the phase transition temperatures for frog and squid photoreceptor membranes, respectively. Blazyk and Steim [1] report the phase transition of rat liver mitochondria and derived liposomes to be around 0 °C. Membranes of *M. laidlawii* grown on 18:2 or 18:1 exhibit phase transition temperatures of −20 and −15 °C, respectively [18]. Living *w/t E. coli* grown at 37 °C exhibit a broad phase transition from −10 °C to growth temperature [25]. Thus many biological membranes exhibit gel-liquid crystal phase transitions which occur well below the temperature range of biological activity, and the calorimetric studies of *Neurospora* phospholipids are consistent with these findings. (d) As evidenced by results of Table 2 and Fig. 2, ergosterol does decrease the heats of transition of phospholipids extracted from both *w/t* and *cel⁻* (Tw 40) organisms.

Intact *Neurospora* do not exhibit a low temperature gel-liquid crystal phase transition. In view of the high ergosterol (rather than cholesterol) content in the *Neurospora* plasma membrane [23] it would be attractive to propose that ergosterol abolishes the heat of transition (ΔH) of intact



low-temperature gel-liquid crystal phase transitions. Calorimeter sensitivities (range) indicated as mcal/sec and scanning rates indicated as °/min

Neurospora membranes as cholesterol does for synthetic phospholipids and biological membranes [16, 8, 26]. The DSC data herein reported demonstrate an ergosterol-mediated reduction in the heats of transition of Folch-washed phospholipids. However, ergosterol in concentrations as high as 100 mole percent does not abolish the heat of the gel-liquid crystal phase transition, whereas concentrations of cholesterol from 33 to 50 mole percent have been found to abolish the ΔH in other systems [21, 10, 26, 15]. Moreover, *Neurospora* phospholipids require a Folch wash to exhibit a gel-liquid crystal phase transition in the DSC. The Folch washing procedure appears to remove some component, possibly nucleotides, from isolated *Neurospora* phospholipids which masks the gel-liquid crystal phase transition. For *Neurospora*, the demonstration of a phase transition, and a narrow range for that transition has been shown to be dependent upon the purity of the extracted phospholipids. Other workers [4, 25] have also found relatively narrow based phase transitions for pure phospholipids and broader based phase transitions for biological membranes.

The endothermic peak occurring at approximately 30 °C in *w/t* and *cel⁻* (Tw 40) whole cells and their extracted phospholipids, is attributable to some change in lipid phase occurring after the major gel-liquid

crystal phase transition. This is the first report of such an occurrence. Endothermic events preceding the gel-liquid crystal phase transition have been reported for other systems [4], and termed pre-transitional peaks. There have been reports of irreversible endothermic events occurring after the gel-liquid crystal phase transition at 60 °C. However, such events are attributed to irreversible protein denaturation [25]. Clearly the peaks exhibited by *Neurospora* cells and derived phospholipids are not irreversible protein denaturation; they occur at 30 °C (a temperature below that of protein denaturation), and they are reversible. Even though these peaks are unexplained, their occurrence in both whole organisms and extracted phospholipids imply similarities of phospholipid structures and behavior, thereby increasing confidence in the use of extracted phospholipid/water systems as accurate model systems for studying the properties of lipids in biological membranes.

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