AC Calorimetry of Dimyristoylphosphatidylcholine Multilayers: Hysteresis and Annealing near the Gel to Liquid-Crystal Transition†

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ABSTRACT: The gel to liquid-crystal phase transition in dimyristoylphosphatidylcholine liposomes was studied with 0.4-Hz ac calorimetry. The ac heat capacity on heating scans exhibited a peak in the vicinity of 23.9 °C with a full width at half-maximum of 0.15–0.20 °C. The enthalpy change was 4.8 kcal/mol, in good agreement with conventional differential scanning calorimetry. On cooling scans, the peak shifted to lower temperature by 0.1–0.5 °C, the width increased to 0.25–0.40 °C, and the apparent enthalpy change was only 40% of that observed on heating. Both the heating and cooling heat capacities were stable for at least 20 min in quasi-isothermal conditions. Following a 1 h anneal at 10 °C, the heating scans were quite reproducible. The results have been interpreted in terms of the nucleation and subsequent annealing of small ordered domains in the bilayer on freezing the acyl chains. No peak associated with the pretransition was observed, as expected since the relaxation time for the degrees of freedom that produce the pretransition is much longer than the period of the 0.4-Hz temperature wave.

In this paper, we report an investigation of the gel to liquid-crystal phase transition (main transition) in multilamellar aqueous suspensions of synthetic dimyristoylphosphatidylcholine (DMPC)[1] by using ac calorimetry. This technique allows the measurement of small heat capacities quasi-isothermally as well as in both heating and cooling scans. Because this is a nonperturbing technique of considerable flexibility, it could be expected to detect the small hysteresis in this phase transition which would arise from superheating and/or supercooling if the phase transition were of first order. Not only does this system exhibit hysteresis but also the apparent enthalpy change on cooling through the transition is significantly less than is obtained on heating through the transition. We have attempted to characterize the state achieved on cooling and suggest its relation to the long-range molecular organization of the bilayer.

Experimental Procedures

Phospholipid Dispersions. DMPC (lot no. 810031) was obtained from Calbiochem. This preparation was found to be homogeneous by thin-layer chromatography and was used without further purification. The suspensions were prepared by heating the lipid in water to a temperature above the Krafft temperature and then shaking the mixture to disperse the lipids. The suspensions were then degassed, reheated to above the transition temperature, and held overnight in an ice bath. The samples were subsequently transferred to the calorimeter vessel and held at 10 °C for 1 h before beginning the initial scan.

AC Calorimetry. Although ac calorimetry has been widely applied to the study of phase transitions in solids, it has only recently been applied to biological systems (Tanasijczuk & Oja, 1978; Jain & Dixon, 1978). Briefly, the technique consists of measuring the thermal response of a sample to an oscillating heat signal. This response is in the form of a temperature wave propagating through the sample.

The sample of heat capacity C is connected through a thermal resistance R to a heat bath at temperature T_0. R is chosen so that the sample-to-bath relaxation time $\tau_b = C/R$ is long in comparison to the period of the temperature wave. Provided the heat path through the sample is smaller than a characteristic length $l_0 = (2\eta/\omega)^{1/2}$, where η is the thermal diffusivity, the ac component of the temperature is uniform across the sample, and its amplitude is related to the heat capacity by

$$T_{ac} = \frac{\dot{Q}_0}{2\omega c} \tag{1}$$

where $\dot{Q}_0$ is the amlitude of the input power. Sullivan & Seidel (1968) discuss the leading corrections to this expression for finite relaxation times to the sample for the heater and thermometer. One may obtain the heat capacity as a function of temperature by varying T_0. Since the signal T_{ac} is independent of T_0 (t) provided the scan rate is slow, it is as easy to measure the heat capacity on cooling as on heating. It is likewise possible to monitor the isothermal time dependence of the heat capacity with this technique.

The major experimental difficulty encountered in the application of ac calorimetry to liquid samples arises from the low thermal diffusivity encountered in liquids. This is typically 1–2 orders of magnitude smaller than is found in crystalline solids and limits the characteristic thermal length l_0. For sample, in water, the thermal diffusivity $\eta \approx 1.5 \times 10^{-3}$ cm^2/s yielding $l_0 = (5 \times 10^{-2}/\omega^{1/2})$cm. At a frequency of 1 Hz, this gives $l_0 = 0.2$ mm. This is to be compared with a characteristic length of 5 mm in aluminum at the same frequency. Uniform heating of the liquid can be assured only if very thin samples ($\sim$10–50 μm) or long periods ($\sim$1–10 min) are used. Calorimeters operating in each of these extremes have recently been described (Tanasijczuk & Oja, 1978; Schantz & Johnson, 1978).

Some of the problems that attend the use of a single thin layer for the sample such as poor seals, large corrections for the heat capacities of heater, thermometer, sample chamber and other addenda, and/or variations in the thermal path length due to fluctuations in ambient pressure or thermal expansion and also the inconveniently long data acquisition

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; T_m, temperature of maximum specific heat at the phase transition; $\Delta T_{1/2}$, the full width at half-height of the specific heat maximum.

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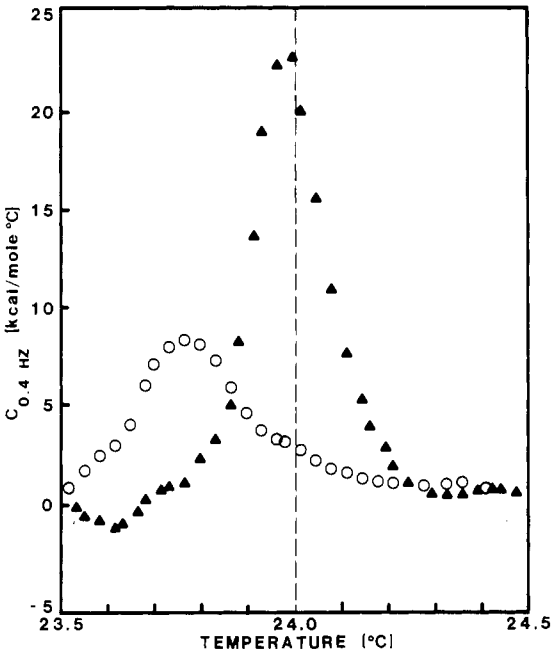

FIGURE 1: Excess 0.4-Hz ac heat capacity of dimyristoylphosphatidylcholine multilayers on heating through T_m at 10×10^{-3} °C/s (▲) and on cooling through T_m at the same rate (○).

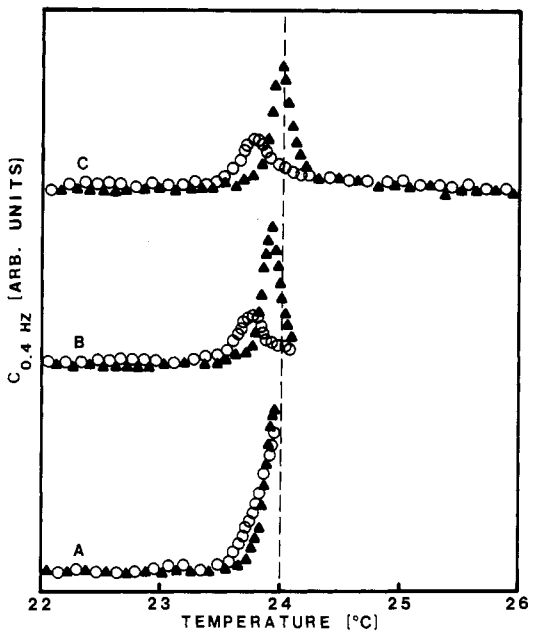

FIGURE 2: Excess 0.4-Hz ac heat capacity of DMPC multilayers annealed at 10 °C for 1 h, heated (▲) to a maximum temperature in or above the transition region, and immediately cooled (○). Run C was annealed at 10 °C for 3 h before heating and cooling began at 27.3 °C.

times of the long-period method may be avoided by the use of a differential instrument. In this study, we have used a differential ac calorimeter employing an aluminum cell in which the liquid sample is arranged in a number of layers, each with a thickness that is small compared to l_0. These are arranged so that the limiting characteristic thermal length is that of the aluminum rather than of the liquid sample. The cell is rigid, easy to seal, and holds a larger volume than can be accommodated in a single layer appropriate to frequencies near 1 Hz. Data acquisition is thus rapid, and the heat capacity of the addendum is removed by the use of a differential reference cell. Details of the instrument will be described in another place.

Results

Because of the equivalence in operation of our ac calorimeter in heating and cooling scans, we initiated a study of the main phase transition in DMPC to search for hysteretic effects that would demonstrate unequivocably that this transition is a first-order, isothermal phase transition. Superheating or supercooling is possible only if the slope of the relevant thermodynamic potential changes discontinuously across the phase boundary. Some typical results are shown in Figure 1 for an 8-μL sample of a 100 mg/mL suspension. On heating a sharp, only slightly assymetric peak with a full width at half-height, $\Delta T_{1/2} = 0.17$ °C was observed at $T_m = 24.00$ °C. This is in excellent agreement with results reported for conventional differential scanning calorimetry (DSC) (Mabrey & Sturtevant, 1976; Lentz et al., 1978). The temperature oscillations had a frequency of 0.4 Hz and an amplitude of 0.5×10^{-3} °C. The mean temperature of the sample was scanned at 1×10^{-3} °C/s from 10 °C where the sample had been annealed for 1 h to a maximum temperature of 27.6 °C. The sample was then immediately cooled at the same rate. As can be seen from Figure 1, the profile of the peak obtained in the cooling scan is markedly different from that obtained on heating. There is not only a hysteretic shift to lower temperatures ($T_m = 23.78$ °C) defining the first-order nature of this transition but also a larger $\Delta T_{1/2} = 0.26$ °C and smaller height. Moreover, the apparent enthalpy change upon cooling is only 62% of that observed on heating. It is clear that despite equivalent external constraints the system has followed a thermodynamic path on cooling different from that taken in the heating scan.

For characterization of the differences between the phase transition observed on heating and that which occurs on cooling, the effect of annealing the sample at various temperatures has been studied. Figure 2 shows a series of scans in which a sample has been annealed at 10 °C for 1 h before heating to various maximum temperatures and immediately cooling. One notes that when the sample is heated to a temperature less than $(T_m)_{heating}$, the cooling curve resembles the heating curve, as in parts A and B of Figure 2. The apparent enthalpy change is slightly larger than that observed on heating in this situation. However, this difference is not larger than that observed over such a limited temperature range in successive heating runs on the same sample. The situation changes drastically when the sample is heated through the midpoint of the phase transition. Now the peaks of the cooling curves are shifted to lower temperatures, and the apparent enthalpy change is significantly smaller than on heating, as seen in curve C of Figure 2. The apparent enthalpy change between 23 and 25 °C measured in the heating scan of curve E is 4.8 kcal/mol, which compares favorably with the values for the enthalpy of transition 5.0–5.4 kcal/mol obtained by DSC (Lentz et al., 1978; Mabrey & Sturtevant, 1976). The apparent enthalpy change for the cooling scan over this same range is only 1.8 kcal/mol. Although the profiles of the specific heat peaks are similar among the heating scans, this is not so among the cooling scans.

Figure 3 presents data obtained on another sample which was annealed for 20 min in the transition region in addition to the 1-h anneal at 10 °C before each heating scan. In curves A, B, and C of Figure 3, this 20-min anneal was carried out at the highest temperature attained in the heating scan, and the temperature was held constant ±0.02 °C for this annealing period. The heat capacity was unchanged from that at the end point of the heating scan throughout the annealing period. Again, the cooling curves that begin from above $(T_m)_{heating}$ give a noticeably smaller apparent enthalpy change than do the

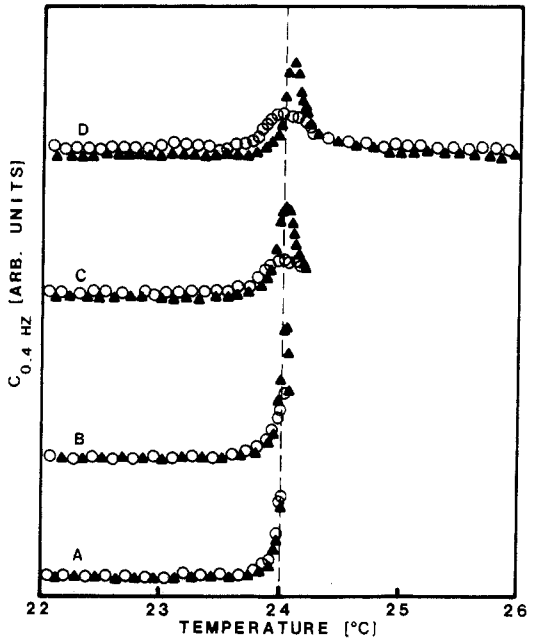

FIGURE 3: Excess 0.4-Hz ac heat capacity of DMPC multilayers. Runs A, B, and C were annealed at 10 °C for 1 h before heating (▲) and for 20 min at the maximum temperature before cooling (○). Run D was immediately cooled.

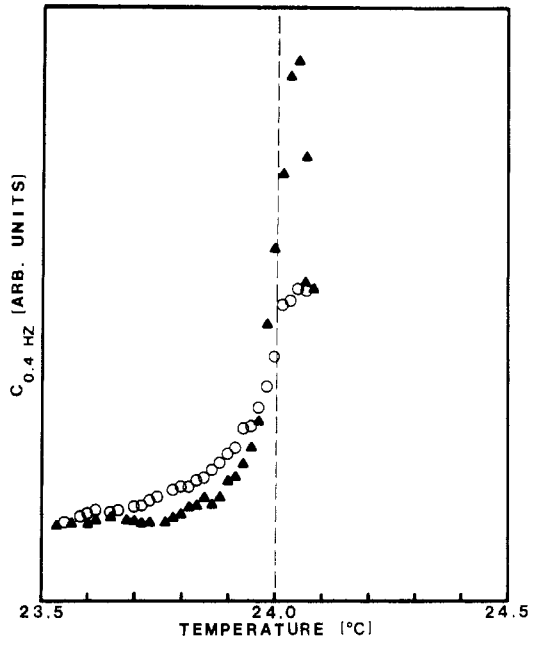

FIGURE 4: Detail of the evolution of the 0.4-Hz ac heat capacity on heating partly through the transition (▲), annealing for 20 min at 24.1 °C, and cooling (○). This is the same as Run B of Figure 3.

corresponding heating scans. The details of run B in the transition region are shown in Figure 4. Comparing runs B and C, one finds that the peak height in run B, the initial heating scan to pass above T_m, is larger than that in the following heating scan C. To ensure that this was not a result of the annealing time, a scan was taken to 27.3 °C and immediately cooled. Both the heating and cooling scans closely reproduced those of scan C. One can also discern a larger initial peak height in the data of Figure 2. This effect is less easily seen there because the final temperature of that scan coincided with T_m.

Both these initial scans were made on samples that had been held below T_m for considerably longer times than in the subsequent heating scans. A scan was made after annealing for

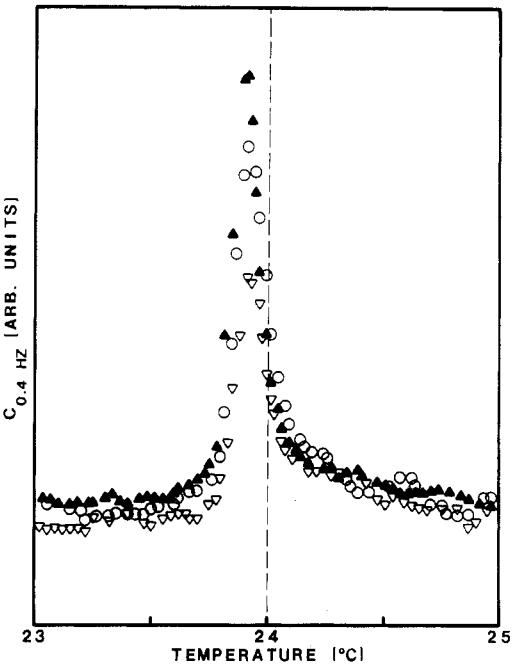

FIGURE 5: Comparison of excess 0.4-Hz ac heat capacity of DMPC multilayers for initial heating (▲), heating following a 1-h anneal at 10 °C (○), and heating following a 1-h anneal at 20 °C (▽). These are runs A, B, and C of Figure 3.

16 h at 10 °C to test whether the increased peak height was due to this different thermal history and not to some deterioration in the sample. The peak height was found to recover about two-thirds of the difference between initial and latter scans. On heating from 20 °C, the peak height returned to that characteristic of a noninitial scan.

In Figure 5 are displayed three successive heating scans: the initial scan, the scan after a 1-h anneal at 10 °C, and the scan after a 1-h anneal at 20 °C. The last temperature lies in the "intermediate" ($P_{\beta'}$) phase between the main transition and the pretransition. No specific heat anomaly associated with the pretransition was observed in any of these experiments, nor should one have been expected when a 0.4-Hz temperature oscillation was used, as discussed below. Figure 5 clearly shows that the height of the peak following the 10 °C anneal is reduced by 15% from the initial scan while the peak following the 20 °C anneal is reduced by 43%. The 10 °C peak height is recovered following a second annealing at that temperature for 1 h.

For investigation of the stability of the states reached on the cooling scans, the temperature of the sample was held fixed (±0.02 °C) for 20 min at representative points in the cooling curve. The measurements exhibited no tendency to recover to the values observed in the heating scans at those temperatures. It would appear from this that although the thermodynamic states traversed on heating and cooling are different and cannot, therefore, both be the equilibrium states of the system, they are nevertheless stable for macroscopic times.

Discussion

The differences between the ac heat capacities obtained on heating and cooling were quite unexpected. Tsong (1974) and Tsong & Kanehisa (1977) have studied the kinetics of the main transition in DMPC by studying the turbidity changes in temperature-jump heating. Their results indicate relaxation times in the range of a few milliseconds to a few seconds for this transition. The stability, or metastability, of different thermodynamic states for macroscopic times at a given temperature would seem to be at variance with such fast relaxa-

tion. Clearly, there are relaxation times associated with freezing the acyl chains that are much longer than those obtained in temperature-jump heating and may indicate that for the ac heat capacity samples the state of molecular aggregation on a different scale from that sampled by turbidity.

In this connection, it is useful to describe somewhat more carefully the properties of the system that are probed by ac calorimetry. For a system as complex as a suspension of liposomes, there are many internal degrees of freedom, some of which may require appreciable lengths of time to equilibrate when the temperature of the system is changed. Let τ_i be the relaxation time associated with such a degree of freedom. If the system is probed at a frequency such that $\omega\tau_i \gg 1$, then that degree of freedom is not affected by the probe. The heat capacity measured at such a high frequency is then no longer the dc heat capacity of the system but is an effective heat capacity $C_{\text{eff}} = C_{\text{dc}} - C_i$. It is for this reason that the pretransition is not observed in 0.4-Hz ac calorimetry; the pretransition has a relaxation time of the order of several minutes (Lentz et al., 1978), and so its heat capacity is not probed by a 0.4-Hz temperature wave. The sensitivity of the calorimeter is sufficient to detect it had it been rapidly relaxing.

Since the enthalpy change measured on heating through the main transition is in good agreement with DSC, a zero frequency experiment, it follows that the principal degrees of freedom associated with this transition have relaxation times on heating that are small compared to the 2.5-s period of our 0.4-Hz temperature wave. This agrees with the observations of the temperature-jump kinetics (Tsong, 1974; Tsong & Kanehisa, 1977) and by a pressure wave method (Clegg et al., 1975). On cooling, the observation of the heat capacity peak, albeit reduced in size, indicates that there must be now some degrees of freedom that relax in a few seconds or faster. The persistence of the reduced heat capacity for a 20-min anneal requires that there are other degrees of freedom associated with this phase transition that have relaxation times of the order of hours at 24 °C. This persistence cannot be explained by a shorter relaxation time of the order of the period of the temperature wave and only partial sampling of the heat capacity of some degree of freedom. Further, at least a portion of these long relaxations remains operative in the ordered phase since the heat capacity does not fully recover its initial peak value on subsequent reheating.

These observations are consistent with a difference between the state of molecular aggregation in an annealed sample and that attained immediately upon freezing the acyl chains. It is plausible that the time required to disorder a cooperative unit of a molecules might be a great deal shorter than is needed to reestablish an ordered unit of similar size. One expects that on cooling many nucleation sites will form simultaneously and may result in a large number of microdomains as compared to the smaller number of larger cooperative units in a well-annealed sample.

An estimate of the average size of a cooperative unit may be obtained by a conventional two-state van't Hoff analysis of the heat capacity near the phase transition (Mabrey & Sturtevant, 1978). For the annealed heating scans, this yields 600–800 molecules per cooperative unit by using the observed apparent enthalpy changes. When this enthalpy observed on heating is taken as representing the ultimate enthalpy change on cooling as well, then the average cooperative unit in the vicinity of T_m while cooling is found to range from 200 to 450 molecules. One expects a distribution of sizes of cooperative units, of course, with both larger and smaller aggregates being present. While the representation of the system by two states in the van't Hoff analysis is clearly an oversimplification, the results, nevertheless, indicate both the trend in the size of the cooperative aggregates and the order of magnitude of their expected size.

If one identifies the cooperative units with domains within the bilayer, a considerable fraction of the lipids in each domain will lie on the domain boundary. In a 200 molecule domain, these boundary lipids can be expected to comprise 25–30% of the total, while in an 800 molecule domain, only 10% would be expected on the boundary. Immediately after freezing, such domains would differ in molecular orientation. Since their reorientation to form larger domains requires the collective motion of many molecules, this should be a much slower process than the melting of such a domain upon heating. Until this reorientation is accomplished, the boundary lipids will not have fully returned to the ordered state and, hence, present a reduced effective heat capacity.

As this discussion indicates, calorimetry is especially sensitive to changes in the size of the cooperative unit and to the large-scale organization of the bilayer. On the other hand, molecular probes used in spin resonance or fluorescence measurements tend to sample preferentially the local environment of the probe. If these probes have no preferred location in ordered or disordered regions, they sample, on the average, the long-range organization of the bilayer as well. However, such probes may produce small distortions in the packing of the bilayer and, thus, serve to nucleate the phase transition. Therefore, they would tend to be in the most rapidly ordering regions and be insensitive to the long-range organization of the bilayer. When used to study kinetics, they probe the initial and rapid processes that establish short-range order, but the slower processes of the formation of large cooperative units may well be invisible to them.

The presence of domains within the bilayer has been directly observed by Hui & Parsons (1975) with wet-stage electron microscopy. Deuterium NMR experiments (Davis, 1979) show the coexistence of fluid and ordered phases in dipalmitoylphosphatidylcholine when the samples are cooled through T_m. This mixed-phase state is reported to persist for at least 30 min and suggests a relaxation time for the dissipation of this state of the same order as we infer calorimetrically for DMPC.

The existence of density fluctuations in the membrane, whether static or dynamic, may be one of the most significant results of the phase transition from a biological standpoint. Papahadjopoulos et al. (1973) have studied the passive diffusion of $^{22}Na^+$ from phospholipid vesicles and found a dramatic increase in the diffusion rate near T_m. Similar effects have been observed in valinomycin-mediated K^+ conductivity (Wu & McConnell, 1973), enzyme transport (Op den Kamp et al., 1974), and ANS transport (Tsong, 1975). Jacobsen and Papahadjopoulos (1976) suggested that density fluctuations near T_m may account for this behavior. Nagle & Scott (1978) showed that critical fluctuations in the lateral density that would occur near a higher order phase transition lead to an anomalously large lateral compressibility of the bilayer. This would facilitate both the insertion of small molecules into the membrane and their passage across it. It would also aid conformational changes in the proteins responsible for active transport in biomembranes.

In view of hysteresis found in the present experiments, the main phase transition in DMPC is first order, not higher order. This is supported by the observation of Albon & Sturtevant (1978) that the analogous phase transition in dipalmitoylphosphatidylcholine multilayers narrows when highly purified

lipids are used. This suggests that the more slowly evolving domains and their boundary lipids may be responsible for the enhanced diffusion near T_m. It is known, however, from monolayer experiments (Horn & Gershfeld, 1977) that the line of first-order monolayer transitions terminates in a critical point. It is quite possible that the bilayer system is sufficiently close to a critical point that critical fluctuations smaller than the average domain size exist in the bilayer. The estimates obtained here of the size of cooperative units allow fluctuations large enough for enhanced transport. Thus, the present experiments do not by themselves exclude either of these possibilities.

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