Biochimica et Biophysica Acta, 688 (1982) 116–122
Elsevier Biomedical Press

BBA 71175

FUSION AND LIPID EXCHANGE IN VESICLES CONTAINING LIPOPHILIC SPIN LABELS

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(Received November 11th, 1981)

Key words: Lipid exchange; Lipophilic spin label; Membrane fusion

Lipophilic non-electrolyte spin labels greatly accelerate the fusion of unilamellar vesicles of dipalmitoylphosphatidylcholine when the system is maintained below the lipid phase transition. Differential scanning calorimetry and centrifugation measurements show that the transformed vesicles are large and probably unilamellar. Differential scanning calorimetry and fluorescence depolarization measurements were also carried out on mixtures of labeled dipalmitoylphosphatidylcholine vesicles and of vesicles composed of pure dimyristoylphosphatidylcholine. A mixing of the membrane components is observed when the vesicles are incubated above the transition temperature of the two constituent lipids. However, the process does not involve a real fusion of the entire vesicles. An exchange of lipid and label monomers between the two lipid phases seems to occur. These observations are discussed in view of the molecular organization of the spin label within the dipalmitoylphosphatidylcholine matrix below and above the lipid transition temperature.

Introduction

Lipid vesicles have been extensively studied as model membranes and as potential drug carriers [1–4]. In this latter context, a real effort has been undertaken to characterize the conditions which ensure a maximal encapsulation of the drug. However, serious difficulties remain concerning the stability with time of the loaded vesicles. In a preceding study [5], we were concerned with the conditions which determine an optimal encapsulation of small non-electrolyte molecules (M_r 213–410), differing essentially by their hydrophilic-hydrophobic balance. The molecules were spin labeled so that their distribution and their molecular organization within the lipid bilayer vesicles could be followed [5,6]. Although high fractions of lipid soluble spin labels could be incorporated in small unilamellar vesicles, the loaded liposomes seemed to be unstable with time. The present paper is devoted to a systematic study of this problem. It is shown that below the lipid transition temperature (T_c), lipophilic spin label molecules induce the transformation of small unilamellar vesicles of DPPC into larger structures. Moreover, when the labeled small DPPC vesicles are mixed with unilamellar vesicles of DMPC and the mixture incubated at 50°C, a rapid lipid exchange is observed between the two liposome populations. These phenomena are dis-

Abbreviations: DMPC, dimyristoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPH, diphenylhexatriene; C_4, C_8, C_{12} and C_{16}, 4-butyramido-, 4-capramido-, 4-lauramido- and 4-palmitamido-2,2,6,6-tetramethylpiperidine-1-oxyl, respectively; T_c, transition temperature of the bilayer crystalline-liquid crystalline phase transition; $P_{octanol/buffer}$, partition coefficient in the system octanol/buffer.

cussed in conjunction with the results obtained previously concerning the molecular organization of the label within the lipid matrix.

Materials and Methods

Materials. Dimyristoyl-L-α-phosphatidylcholine (DMPC) and dipalmitoyl-DL-α-phosphatidylcholine (DPPC) were purchased from Sigma Chemical Company. The spin labels were synthesized as previously described [5]. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Aldrich. All reagents and solvents were pro analysi products.

Preparation of vesicles. Phospholipid vesicles (multilamellar and sonicated unilamellar) were obtained as described previously [5] using a 100 mM, Tris-HCl(pH 7.4)/0.9% NaCl buffer. Sonication was performed with a Branson sonifier B 12 for up to 20 min. Centrifugations and ultracentrifugations were made on a Beckman model J-21B and model L-2 ultracentrifuge.

Turbidity measurements. Sonicated dispersions were prepared at a lipid concentration of 5.5 μmol/ml. At the end of the sonication, the sample was brought to room temperature (21 − 23°C) within a few minutes and centrifuged for 4 min at 16 000 × *g* on an Eppendorf centrifuge. Absorbance was monitored at 550 nm against a buffer blank, using a Cary 14 spectrophotometer, in 1 cm cells.

Differential scanning calorimetry measurements. Multilamellar or unilamellar vesicles were prepared as described above at a lipid concentration of 55 μmol/ml. For the experiments performed with a mixture of different populations of unilamellar vesicles, sonicated DMPC unilamellar vesicles were maintained in a water bath at 50°C during the sonication of the DPPC vesicles containing the spin label. Samples of sonicated vesicles were then immediately mixed in equal quantities and incubated at the appropriate temperatures. After various time intervals, 100-μl aliquots of lipid suspension were placed in sealed stainless steel sample pans. A reference sample was similarly prepared using 100 μl buffer. Measurements were carried out on a Setaram DSC 111 differential scanning calorimeter (Lyon, France) operating at a heating rate of 2 K/min.

Fluorescence depolarization measurements. The fluorescence depolarization associated with the hydrophobic fluorescent probe DPH was used to monitor the changes in the fluidity of the lipid matrix accompanying the gel-liquid crystalline phase transition. The vesicles were labelled by addition of DPH dissolved in tetrahydrofuran (DPH/lipid molar ratio, 1:500) to the multilamellar suspensions. Experiments were carried out with an Elscint Microviscosimeter model MV-1a (Elscint Ltd., Haifa, Israel). This apparatus is designed to give directly the degree of fluorescence polarization, P, following Eqn. 1:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (1)$$

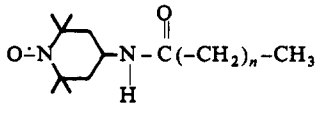
where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicularly to the direction of polarization of the excitation beam, respectively. This apparatus gives P with an accuracy better than 5%. The temperature of the sample was controlled to within 0.5 K. The heating rate was 2.5 K/min.

Results and Discussion

Turbidity measurements which are fast and non-perturbative are frequently used to detect modifications occurring in lipidic suspensions. The turbidity of DPPC suspensions loaded with the labels listed in Table I, was followed by measuring

Table I

Chemical structure, abbreviated name and logarithm of the partition coefficient ($P_{octanol/buffer}$) of the 4-alkylamido-2,2,6,6-tetramethylpiperidine-1-oxyl



Structure	Abbreviated name	log $P_{octanol/buffer}$ [5]
$n=2$	C_4	1.35
$n=6$	C_8	3.36
$n=10$	C_{12}	5.47
$n=14$	C_{16}	7.57

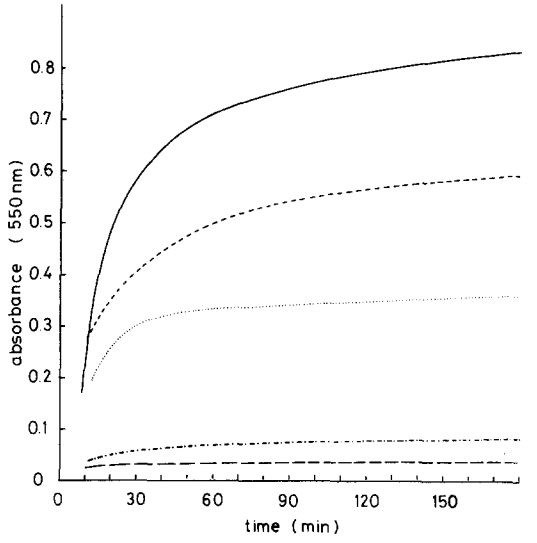


Fig. 1. Turbidity measurements carried out at 23°C on suspensions of small unilamellar vesicles of DPPC. Influence of 20 mol% of the labels listed in Table I. Time zero corresponds to the end of the sonication. Lipid concentration, 5.5 μmol/ml; λ=550 nm. — — —, DPPC alone; ·–·–·–·, DPPC+C_4; ·····, DPPC+C_8; - - - - - -, DPPC+C_{12}; ————, DPPC +C_{16}.

at room temperature the 550 nm absorbance. The results are shown in Fig. 1.

A very slight absorbance increase is detected for the control DPPC vesicles, at least within the 3 h which follow sonication. By contrast, the other curves, which correspond to vesicles loaded with 20 mol% label, reveal important and rapid modifications of the suspension optical properties. These changes can be correlated with the hydrophobicity of the molecules as shown in Fig. 2A where the absorbance values after 3 h, i.e., near equilibrium (Fig. 1) are plotted as a function of the logarithm of the partition coefficient of the labels (Table I). The perturbation induced by the probes on the stability of the vesicles is proportional to its penetration in the bilayer. Fig. 2B shows the influence of the C_{16} concentration on the stability of the vesicles. At 1 mol%, no effect is measured, but at 5, 10 and 15 mol% significant perturbations are detected; when the concentration reaches 20 mol%, an unexpected rapid rise is observed. This 'critical' concentration has been observed previously in the ESR measurements. Above 15%, the properties of the entire phosphatidylcholine phase are influenced by the presence of spin probe clusters [6].

The optical measurements described in Fig. 1 and 2 do not allow us to draw any definite conclusion concerning the process which is taking place nor concerning the properties of the system at

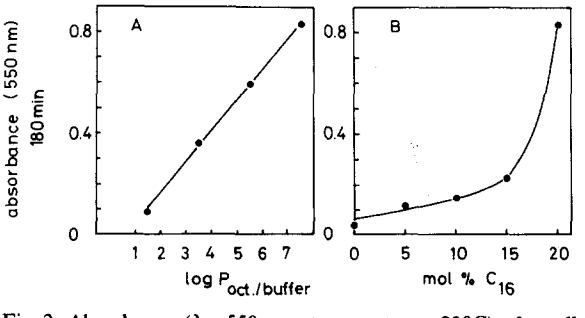


Fig. 2. Absorbance (λ=550 nm, temperature=23°C) of small unilamellar vesicles of DPPC measured 3 h after sonication. Lipid concentration: 5.5 μmol/ml. A. Influence of the hydrophobicity of the label (*P* is the octanol/buffer partition coefficient of the labels). The label concentrations are the same than in Fig. 1. B. Influence of concentration of C_{16} on the absorbance reached at equilibrium.

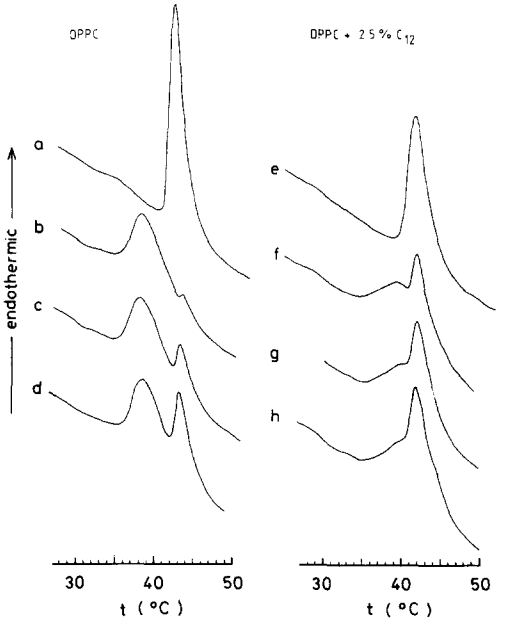


Fig. 3. DSC scans observed with pure DPPC vesicles (left side) and with DPPC vesicles labeled with 2.5 mol% of C_{12} (right side). The lipid concentration is 55 μmol/ml. Temperature= 23°C. a, e, multilamellar vesicles; b–h, unilamellar vesicles scanned: (b, f) 0.25; (c, g) 1; (d) 24; and (h) 3.5 h after sonication.

equilibrium. Other experimental techniques are obviously needed to differentiate among several possible pathways such as aggregation or fusion.

Differential scanning calorimetry (DSC) provides valuable information on the thermotropic properties of lipid vesicles [7 – 9].

Fig. 3 describes the calorimetric properties of pure DPPC vesicles and of vesicles containing 2.5 mol% C_{12}, respectively. Fig. 3a shows the pattern characteristic of multilamellar DPPC vesicles: a small pretransition at 35.5°C and a highly cooperative transition at 42.2°C. When C_{12} is present, the pretransition vanishes and the peak of the main transition becomes broader and smaller; the transition temperature being lowered to 41.7°C (Fig. 3e). This latter value diminishes with the label concentration. With 10 mol% of C_{12}, the transition occurs at 40°C.

After sonication, the DSC pattern corresponding to pure DPPC is composed of a broad peak centered at 38.1°C (Fig. 3b). However, besides the main transition, a shoulder is noticeable at 43°C. If the suspension is kept at 20°C, this shoulder increases gradually with time (Fig. 3b → 3d). This process does not occur at 50°C over the same incubation period (data not shown). In presence of C_{12} (Fig. 3, right column) the appearance and growth of the 42°C peak is very much faster. 15 min after sonication, the peak corresponding to small sonicated vesicles, centered at 39.5°C, is barely resolved (Fig. 3f) and 3.5 h after sonication it has almost vanished (Fig. 3h). This rapid modification is also temperature dependent since it is not observed when the DPPC-C_{12} suspension of unilamellar vesicles is maintained at 50°C.

It must be recalled that the presence of the label does not prevent the formation of unilamellar vesicles; this was demonstrated previously by Sepharose 4B chromatography [5].

The process described in Fig. 3 is irreversible and if the C_{12} concentration is larger than 2.5 mol%, its kinetics increase rapidly. At 10 mol%, the peak corresponding to small sonicated vesicles is already undetectable 15 min after sonication (data not shown). The peak displacement from 39.5 to 42°C is highly suggestive of a modification of the bilayer curvature [10] and accordingly, the alterations of the optical properties presented in Fig. 1 are not related to an aggregation process. It remains however that the transformation might lead either to the formation of large unilamellar or to the reconstitution of multilamellar vesicles. In order to differentiate between these two possibilities, ultracentrifugation experiments were undertaken. Multilamellar vesicles, with or without 2.5% C_{12}, sediment at very low speed ($2500 \times g$; 15 min; 23°C). In the case of the highly turbid suspensions of sonicated vesicles containing the label, a pellet is observable only after high-speed centrifugation ($130000 \times g$; 30 min, 10°C). This rules out the hypothesis of a return of the vesicles to the multilamellar state.

The transformation of the small unilamellar vesicles of DPPC, which occurs over long periods of time, has been described previously by Schullery et al. [11]. These authors have demonstrated that below T_c, the vesicles tend to form slowly but spontaneously larger entities of about 70 nm diameter. The DSC pattern becomes similar to that of multilamellar but without pretransition [12]. This corresponds to our observations (Fig. 3); the presence of hydrophobic molecules markedly increases the slow spontaneous fusion of unilamellar vesicles of DPPC into larger unilamellar entities. Usually, the turbidity changes induced by the presence of 'foreign' molecules in the lipid matrix have been associated with the formation of multilamellar vesicles [13–15]. Obviously, this is not the case in our experiments. The spin label molecules have a catalytic activity on the fusion of small unilamellar vesicles of DPPC maintained at 20°C.

The use of one lipid population limits considerably the possible conclusion concerning the mechanism of interaction. In order to investigate other mechanisms such as lipid exchange, experiments were undertaken with mixtures of vesicles populations.

Small sonicated vesicles made of pure dimyristoylphosphatidylcholine (DMPC) molecules were mixed in equimolar concentration with small sonicated DPPC vesicles containing the labels. The mixtures were analyzed both by DSC (Figs. 4 and 5) and by fluorescence depolarization (Fig. 6). The DSC scan observed immediately after mixing of equal amount of pure DMPC and pure DPPC vesicles is shown in Fig. 4a. The transition of each population of vesicles is apparent at 19°C [9] and 38°C. The DSC results obtained in the presence of

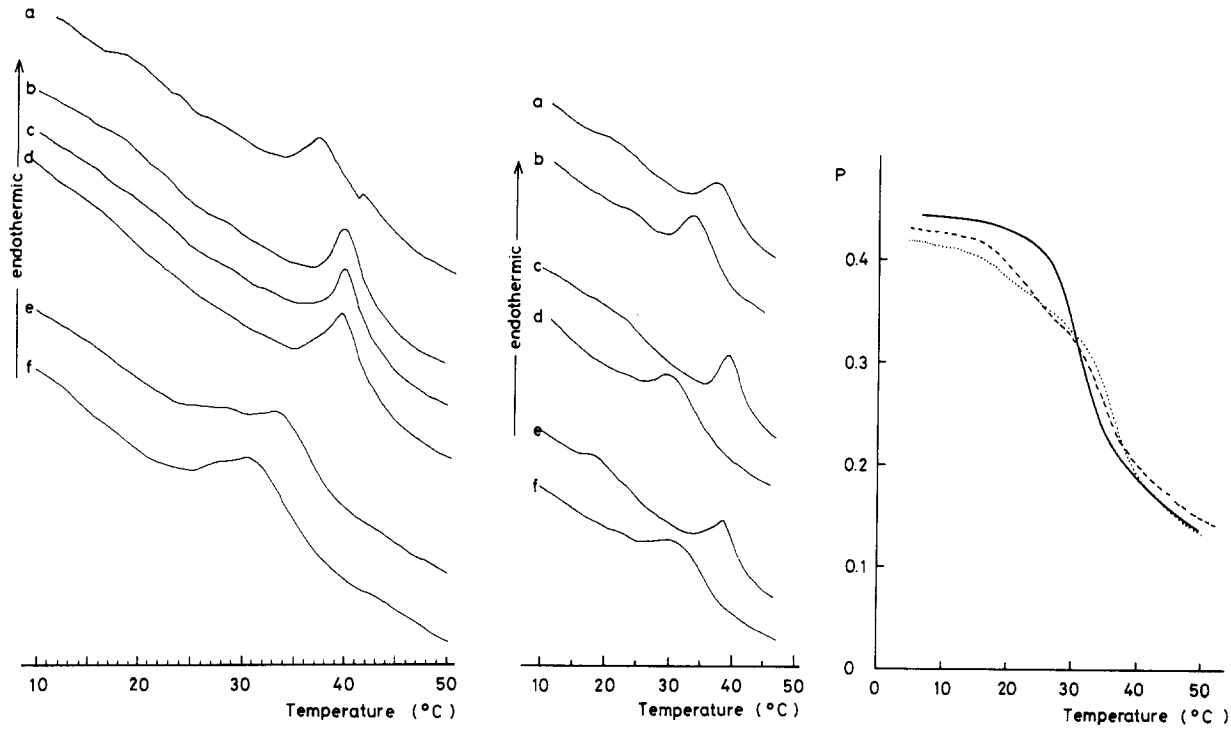


Fig. 4. DSC measurements carried out with sonicated vesicles of pure DMPC (55 μmol/ml) mixed in equimolar concentration with: a, sonicated vesicles of pure DPPC. The mixture was incubated 15 min at 20°C before DSC recording. b–f, sonicated vesicles of DPPC labeled with 20 mol% C_{12}. Incubation at 20°C for 15 min (b), 1 h (c) or 3 h (d). Incubation at 50°C for 1 h (e) or 3 h (f). No significative change of the liposome suspension which did not contain the label (profile a) was observed in the same experimental conditions.

Fig. 5. DSC measurements recorded with suspensions of DMPC sonicated vesicles (55μmol/ml) mixed in equimolar concentration with: sonicated vesicles of DPPC labeled with 20 mol% C_4. Incubated 4.5 h at 20°C (a) or 3 h at 50°C (b); sonicated vesicles of DPPC containing 20 mol% C_8. Incubated 4.5 h at 20°C (c) or 3 h at 50°C (d); sonicated vesicles of DPPC containing 20 mol% C_{16}. Incubated 21 h at 20°C (e) or 3.5 at 50°C (f).

Fig. 6. Temperature dependance of fluorescence depolarization (P) of DPH solubilized in unilamellar vesicles. The vesicles were purified by Sepharose 4B chromatography. The liposome mixture, composed of sonicated vesicles of pure DMPC (55 μmol/ml) mixed in equimolar concentration with sonicated DPPC vesicles containing 20 mol% C_{12}, was chromatographed just after mixing (·····), after 4 h incubation at 4°C (- - - - -) or after 4 h incubation at 50°C (————).

20 mol% C_{12} are shown in Fig. 4b–f. Fig. 4b shows that the kinetics described previously concerning the transformation of the sonicated DPPC vesicles are indeed very fast at high label concentrations. 15 min after sonication, only the 40°C peak is observed. Fig. 4b–d shows that when the lipid mixture is maintained for 3 h at 20°C, the DSC pattern remains virtually unchanged; at this temperature, DMPC is in the phase transition. Identical results were observed at 4°C when both types of vesicle are rigid (data not shown).

On the other hand, if the mixture is incubated at 50°C (Fig. 4e and f), the peaks associated with the two lipids tend to shift towards each other. After 3 h incubation, a single broad transition centered at 30°C is detected.

Fig. 5 presents the DSC profiles in the case of 20 mol% C_4 (Fig. 5a, b), C_8 (Fig. 5c, d) and C_{16} (Fig. 5e, f), respectively. At 20°C, no shift of the peaks occurs after several hours (Fig. 5a, c, e), whereas at 50°C, the mixing kinetics increase drastically with the hydrophobicity of the label

molecules (Fig. 5b, d, f). Similar results were obtained with the label introduced in DMPC vesicles instead of DPPC.

These results strongly suggest that a mixing of the lipid phase is taking place between the two lipid populations. This exchange phenomenon should be clearly differentiated from a true fusion process since in this latter case, a new intermediate peak should indeed progressively appear but the original transition should remain centered at their original positions [8]. This is clearly not the case in our experiments.

The transformed vesicles are characterized by a mixed lipid phase as demonstrated by fluorescence depolarization measurements. Fig. 6 shows the results obtained with the fractions corresponding to the small unilamellar vesicles after the Sepharose 4B chromatography. If the chromatography is carried out after mixing of the two vesicle populations, at 20°C, the transitions appear at 19°C (DMPC) and 39°C (DPPC-C_{12}) (dotted curve). Similar results are obtained if the vesicle mixture is incubated 4 h at 4°C prior to chromatography (dashed curve). On the other hand, if the incubation is performed at 50°C, the fluorescence depolarization measurements corresponding to the unilamellar vesicles fraction show a monophasic transition (solid curve); the temperature of the midpoint, 30°C, is intermediate of the two lipid transitions.

In summary, we have shown that when the spin labels are introduced in sonicated DPPC vesicles, two types of phenomenon may occur, depending on the temperature of the experiment. Below T_c, the small sonicated DPPC vesicles containing the lipophilic spin labels undergo a fusion process leading to the formation of large and probably unilamellar vesicles; this process remains unchanged when sonicated DMPC vesicles are present in the fluid or rigid state.

Above 42.2°C, the stability of the small DPPC vesicles is modified by the label. The membrane properties are perturbed since the presence of the label triggers a rapid molecular exchange between the labeled vesicles of DPPC and those of DMPC. This exchange might imply a diffusion of lipid and label monomers through the aqueous phase.

It seems worthwhile to consider the results in the context of our previous findings concerning the molecular organization of these spin labels in model membrane matrix. Chatelain et al. [6] have proposed a model following which below the DPPC phase transition, the membrane model can be viewed as a mosaic structure composed of spin label clusters. Above the DPPC phase transition, the C_{16} spin label molecules diffuse freely in an homogeneous fluid DPPC bulk phase.

In the framework of the present study, the presence of the label cluster seems to destabilize the membrane structure in such a way that fusion becomes more favorable. The defects that the presence of the clusters create in the DPPC rigid lattice are probably the prerequisite for the catalytic action of the labels on the fusion process.

This finding can be compared with the fusion process that myristic acid induce in unilamellar DMPC [16] or DPPC vesicles [17] when the systems are incubated in the range of the phase transition temperature. Hauser et al. [18] have shown that long chain fatty acids cluster in fluid phospholipid bilayers at physiological pH. The importance of the formation of unstable domains in the lipid matrix (perturbed matrix) in the fusion process has been emphasized by Papahadjopoulos in several papers (for a review see Ref. 19).

Above the phase transition, the properties of DPPC are altered, although the label molecules diffuse randomly within the bulk lipid phase. This modification is such that it leads to a destabilization of the membrane structure which in turn might produce an enhancement of the diffusion of lipid monomers in the aqueous phase.

It can be concluded that, although the lipid vesicle formation is perfectly feasible with high concentration of non-electrolyte hydrophobic molecules, the stability of the small vesicles is seriously compromised. The preparation of liposomes with lower bilayer curvature [20] could be a possible way to overcome this difficulty.

Acknowledgments

F.D.-Q. thanks I.R.S.I.A. (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture) for financial assistance.

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