LIPOSOME BILAYER MODEL SYSTEMS OF FREEZING LIVING CELLS

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Received 25 May 1971

1. Introduction

The results of freezing studies on plant tissues and other living cells increasingly point to membranes in cell surfaces and cell organelles as principal sites of freezing injury [1–6]. The large increases in the amounts of phospholipid and lipoproteins found to be associated with the extreme adaptation of some tree cells to freezing in winter has also focussed attention recently on the importances of membranes in freezing phenomena [7]. The nature of the molecular mechanisms whereby injury and tolerance to freezing is produced is, however, still uncertain [7]

Since there is increasingly a belief that cell membranes may contain regions of lipid in a bilayer form, we felt that an examination of the effects of freezing and thawing on phospholipid liposome systems might be useful as a model system leading to an understanding of freezing and thawing injury to living cells.

Because of the large number of microscopic observations which have been made on the freezing of living cells, we examine liposomes under the same conditions. We also include some studies of the effects of freezing on the permeability of these liposomes.

An analogy in terms of osmotic and permeability behaviour to living cells has been demonstrated with the multi-bilayer system of liposomes studied by Bangham and co-workers [8, 9].

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2. Experimental

Liposomes were prepared in the conventional manner with some modifications as suggested by Weissman et al. [10]. Twentyfive mg aliquots of egg yolk lecithin prepared by the method of Dawson [11] and dissolved in chloroform were dried under a stream of nitrogen while shaken in a 100 ml Erlenmeyer flask so that a thin film of lipid was deposited evenly over the bottom of the flask. The films were dried further in a vacuum desiccator over silica gel for 10 min and then agitated actively with 1 ml of one of the marker solutions so that this solution could be incorporated into the liposomes. Various types of charged and uncharged markers were thus incorporated, e.g. 0.145 M K_2CrO_4, KH_2PO_4, glucose or glycine. Only one marker was entrapped in any one suspension. The suspensions were all stored at 4° for 18 hr to complete swelling. Each suspension with its own marker trapped in the liposomes was then transferred to a length of 5/32″ Visking dialysing casing and permitted to dialyse against 1 l of an equimolar solution of KCl–NaCl (total molarity = 0.145 M), four fresh changes each of 1 l being made every hour until the dialysate was free of marker.

The dialysing sacs containing the liposome suspensions were now suspended in air in 10 ml stoppered test-tubes which were then immersed in an alcohol freezing bath. Temperatures of the liposome suspension during freezing were monitored by a thermistor inserted in one of the suspensions. The suspensions were cooled at the rates of 0.5° a minute until frozen and then cooled further at the same rates to a final temperature of −30° where they were left for a period of 15 min. The tubes and suspension were then

Table 1
Release of markers from liposome suspensions during dialysis for 24 hr after freezing to -30° in (a) dialysing sacs, (b) test-tubes. (Results expressed in micrograms marker released from each liposome suspension containing 25 mg lipid.)

Marker	Phosphate [a]	Chromate [a]	Glucose [a]	Glycine [a]	Phosphate [b]
Control replicate 1	17	60	34	37	21
Control replicate 2	8	55	30	25	31
Freezing replicate 1	198	312	192	83	127
Freezing replicate 2	131	211	113	82	150
Freezing replicate 3	–	–	–	–	119
Freezing replicate 4	–	–	–	–	128

Table 2
Release of chromate marker from liposome suspensions by freezing to −30°. (Each suspension contains 60 mg lipid.)

	Control unfrozen			Frozen		
	Micrograms released	Micrograms residue	% released	Micrograms released	Micrograms residue	% released
Replicate suspension 1	18	522	3.3	344	83	80.5
Replicate suspension 2	11	355	3.0	244	95	71.9
Replicate suspension 3	17	233	6.8	316	114	73.5
Replicate suspension 4	17	272	5.9	266	166	61.6
Average	–	–	4.2	–	–	71.0

thawed at room temperature at which time the sacs with suspension were immersed directly in 5 ml of a fresh equimolar solution of KCl–NaCl (total molarity = 0.145 M) to permit efflux of the markers. Dialysis was allowed to proceed for 24 hr at 4° with one fresh change of dialysing solution being made after 1 hr. The dialysates were poured off at each change and stored for subsequent analysis.

To check the possibility of complications due to effects which freezing might have on the dialysing casing itself, some freezing experiments were performed on suspensions transferred temporarily from the casings to test tubes to permit freezing and thawing to occur after which they were returned to the dialysing sacs to measure efflux as before. Only suspensions containing phosphate markers were tested in this way. The controls were suspensions treated in a manner identically to all the other suspensions except that they were not frozen. The degree of efflux of markers from the liposomes was determined from analysis of the dialysing solutions.

Finally 4 replicate tests were performed using chromate markers in which the liposomes prior to freezing were cleared of external marker solution by three successive centrifugations and resuspensions using 3 ml of fresh KCl–NaCl (1 : 1, 0.145 M) at each resuspension. The centrifugations were each for 10 min at 1500 *g*. Correspondingly marker release by freezing was determined by (1) suspension of the centrifugally cleared liposomes in 1 ml of 1:1 KCl–NaCl (0.145 M) in a test-tube, (2) freezing of the suspension to −30° at rates similar to those used above, (3) thawing in air at room temperatures, (4) centrifugation, and (5) analysis for chromate in the supernatant after centrifugation. In the present experiments 60 mg of egg yolk lecithin were used to prepare each suspension. Also because of lack of uniformity noted in the amount of marker trapped in the various suspensions, probably as a result of variable degrees of agitation in preparation, marker release in the latter experiments was estimated and expressed in terms of percentage of markers released from the liposomes as well as in absolute amounts. Marker in residues left in the liposome after release was determined by extraction of the lipid and chromate into lower and upper phases, respectively, of a

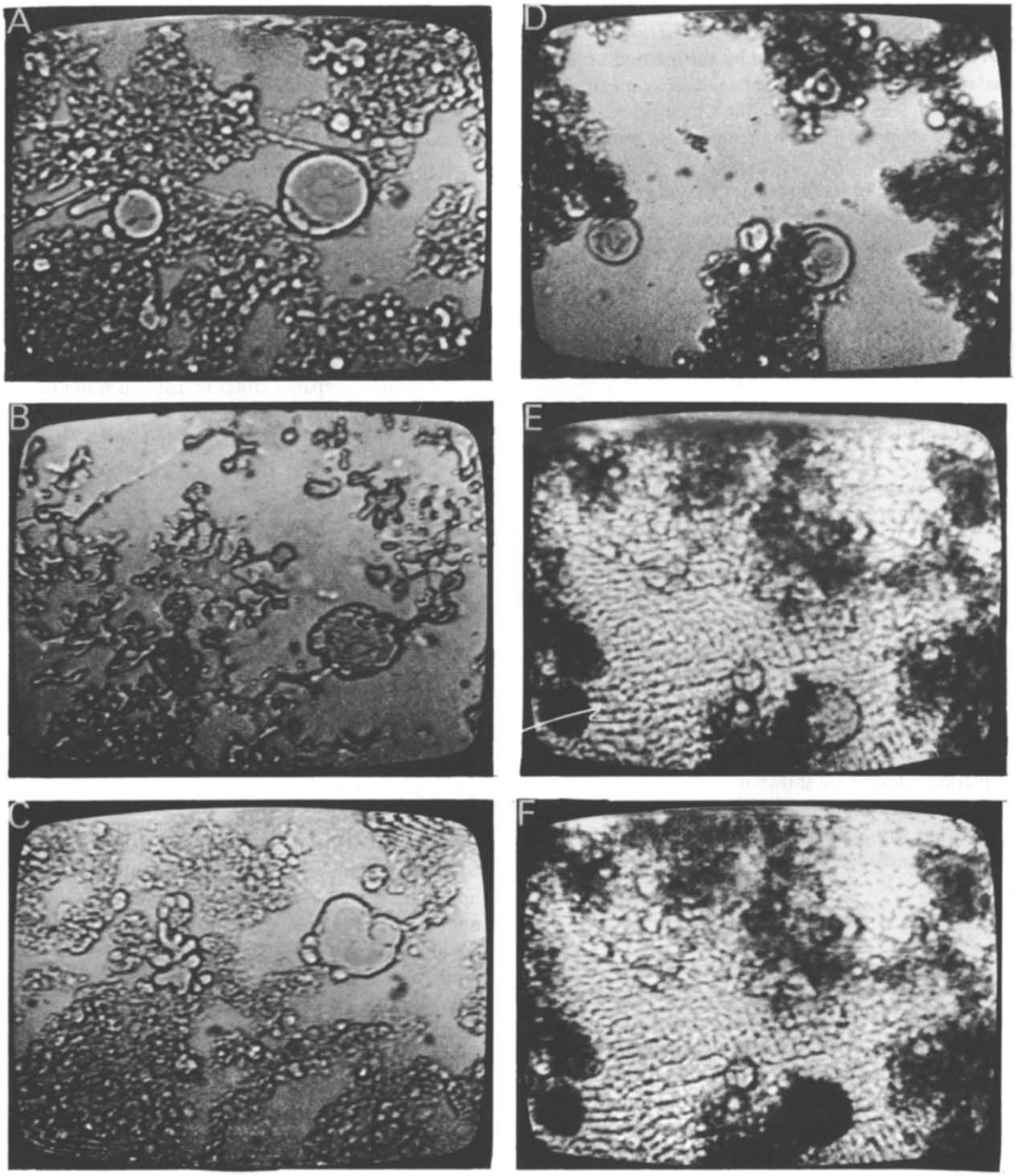

Fig. 1. Microphotographs (× 666) of freezing of liposome suspensions in KCl–NaCl (1 : 1, 0.145 M). *Slow freezing* (0.5° per min): left sequence, A, B, C. (A) liposomes before freezing, (B) frozen liposomes encased in ice at −15°, liposomes contracted and shrivelled, with smallest liposomes almost hidden from view, (C) thawed, swollen liposomes showing irregular conformation and smaller liposomes once again filling the field of view. *Fast freezing* (20° per min): right sequence, D, E, F. (D) liposome before freezing, (E) sudden freezing of mounting medium and penetration of ice into interior of left liposome without contraction, (F) less than a second later, continued progress of ice into second liposome (−50°).

1 : 1 : 0.4 chloroform–methanol–water extract of the liposomes.

Phosphate was determined by the method of Barlett [12], chromate by optical density measurements at 415 nm, glucose by the Glucostat reagent [13] and glycine by the Moore and Stein procedure [14].

The freezing of the liposomes was examined under the microscope. Liposome suspensions prepared as before but in an equimolar solution of KCl–NaCl (total molarity = 0.145 M) or in distilled water were mounted between cover slips in microscope chamber cooled thermoelectrically or with solid CO_2 or liquid nitrogen and monitored with a thermistor. Enough suspension was used to fill at least 1/3 of the field.

Microphotographs of freezing liposome suspensions were obtained by photography of single frame monitor images of videotape recordings of freezing made with Sony television equipment.

3. Results

3.1. *Permeability effects*

The results of two replicate tests with four different markers using dialysis to determine release of markers from the frozen-thawed liposome suspensions and release expressed in terms of amounts of marker released are shown in table 1.

The results of four replicate tests with chromate marker but with release of marker determined from analysis of supernatants after centrifugation are shown in table 2.

3.2. *Microscope observations*

Cooling was at the rate of 0.5° per minute. As soon as ice crystallization was initiated in the mounting solution, the crystals were noted first to traverse the field and thrust between the liposomes or aggregates of liposomes. With progressive crystallization as the temperature dropped, the liposomes were observed to contract markedly and become squeezed between the interstices of the growing ice crystals (fig. 1, A and B). At temperatures of −20° the reduction in liposome volumes was so considerable as to cause some of the smaller ones to be nearly obliterated from the field of view except for the presence of an interlocking network of fissures in the transparent ice mass in which they were lodged (fig. 1B). On thawing, the liposome aggregates swelled visibly and reoccupied nearly the same area of microscopic field as before freezing (fig. 1C). Microscopic examination of the liposomes after freezing and thawing both in ordinary light and with crossed polaroids gave the impression that liposomes which had been frozen had lost some of the smooth and regular features of normal liposomes (fig. 1C).

Freezing was also induced inside the liposomes by hindering equilibration either by suspending the liposomes in water instead of salt solution or by very rapid cooling.

Liposomes prepared either in distilled water or in 0.145 M solution of KCl–NaCl (1 : 1) were mounted in distilled water and cooled at the rate of 0.5° per minute. The course of events observed with both kinds of liposomes suspended in water during the freezing was now quite different from that observed with normal salt-suspended liposomes at this rate of freezing. At the instant of crystallization, ice was observed to proceed into the interior of the liposomes (fig. 1E), in a manner very similar to that observed in the case of rapid or ultra-rapid freezing of living cells generally [4, 18–21].

Individual liposomes or groups of liposomes in succession were observed to undergo visible darkening by internal crystallization (fig. 1E, F) in a manner reminiscent of the "flashing" or "blacking out" observed with living cells frozen internally in this way [4, 20]. An identical pattern of internal freezing behaviour could be obtained even in salt-suspended liposomes simply by cooling very rapidly (20° per minute). Thus internal crystallization of liposomes could be induced at will and this was confirmed by the fact that the liposomes or aggregates of liposomes thus frozen gave no evidence of contraction and retained their original shape and volume while encased in the ice even down to temperatures of −50°. On thawing, ice crystals both in and out of the liposomes simply melted without any marked indication of swelling. As with externally frozen liposomes, there were no microscopically observable indications of damage but bulk experiments with markers will have to be performed to determine whether the same marker release from lipo-

somes occurs with internal freezing as with external freezing.

4. Discussion

The most important question which first had to be resolved was whether liposomes are damaged or affected in some ways by freezing and therefore simulate cell damage. The method for assessing liposome damage which depends on measurement of the release of ions or molecules trapped within the liposomes [10], is identical in principle with that often used to measure the extent of freezing injury in living plant cells [15].

While the results in table 1 obtained by dialysis procedures show considerable variability and relatively large values for the extent of marker release from the unfrozen controls, these results, considered together with the results obtained by centrifugation procedures shown in table 2, clearly indicate that freezing does cause a pronounced increase in the release of both ionic and nonionic markers trapped in the liposomes. The results in table 2 show consistently high values for the percentage of marker released by freezing in four replicate experiments and correspondingly low values for the unfrozen controls. This serves to emphasize the fact that chromate markers once trapped in the liposomes are strongly retained and are released only as a result of damage to the lipid membrane. Such a release upon freezing is similar to that which occurs with other membrane damaging agents [16] when the bilayer structure has been altered.

The progressive reduction in volume of the liposomes observed under the microscope during slow freezing at the rate used in the bulk experiments (0.5° per min) is consistent with the view that liposomes like living cells experience osmotic dehydration in consequence of freezing and concentration of the salt medium in which they are suspended. These effects are analogous to slow freezing, desiccation or plasmolysis of living cells [1, 17, 18]. More rapid freezing of liposomes in salt medium even at rates of 4° per min elicit exactly the same freezing response as is obtained with slow freezing. The permeability of the liposome membranes to water is sufficiently great at these rates of freezing to permit equilibration of the salt solution in the interior of the liposome with the freezing and concentrating salt solution on the outside [18, 21]. The freezing point of the solution in the interior of the liposome is lowered in consequence and crystallization in the interior prevented.

On the other hand where equilibration is not maintained as in slow freezing (0.5° per min) of the liposomes suspended in distilled water or in fast freezing in salt solution (20° per min) internal crystallization occurs as in living cells which have been frozen rapidly. A theoretical value of the critical freezing rate producing internal freezing in the liposome depends on an estimate of the permeability of water through the limiting membranes of the liposomes [21]. The ratio of liposome surface to volume which would also determine the critical freezing rate is difficult to estimate because of the considerable variability in size and shape of the liposomes. In this regard, on some occasions of rapid freezing (20° per min) internally frozen liposomes were observed side by side with liposomes which had escaped internal freezing by equilibration and had undergone dehydration and contraction instead.

In summary these observations on freezing of liposomes indicate that these systems simulate much of the behaviour of living cells. The mechanism whereby the bilayer systems are damaged by dehydrative freezing needs to be examined more closely. The fact that a pure lipid bilayer system uncomplicated by protein can be damaged by freezing may have relevance to the problem of freezing injury and resistance to living cells and suggests that the liposome with different lipids and unsaturation may be worth further study for attempting to reach an understanding of the mechanism of freezing injury.

Acknowledgements

We are grateful to Miss Marilyn Tyson for the preparation of the egg yolk lecithin, to Mr. Douglas Hill for the construction and setting up of the microscope freezing apparatus, to Dr. J.E. Stangroom for the Videotape recordings, and to the Science Research Council for the financial support to D. Chapman.

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