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# Osmoregulation of fusogenic protoplast fusion *

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**Fusion permissive (fusogenic) plant protoplasts have been isolated from wild carrot suspension cultures. Fluorescence, video, and electron microscopy were used to monitor the sequence of fusion events in response to calcium and osmotic stress. Fusion of these protoplasts was enhanced by calcium and inhibited by EGTA. The plasma membranes of these protoplasts were so fusion permissive that osmotic stress, in the absence of external calcium, would stimulate protoplast fusion. Protoplast aggregation but not membrane fusion occurred in a hypertonic solution in the presence of 10 mM calcium. Membrane fusion was not observed until the external osmoticum was lowered. The fusogenic carrot protoplasts provide a model system for studying membrane fusion.**

## Introduction

Fusion of biological membranes is necessary for cellular processes such as secretion, endocytosis, and fertilization. Although membrane fusion is an important and ubiquitous event, the sequence of events by which membrane fusion occurs is not well understood.

We have developed a plant system in which the plasma membranes of protoplasts fuse readily with only a mild calcium stimulus. Specifically, fusogenic protoplasts can be isolated from wild carrot cells grown in suspension culture [1]. Fusion of these protoplasts is enhanced by calcium and inhibition by the calcium chelator, EGTA. The fusogenic protoplasts are approximately 15 $\mu$m in diameter and can be observed easily by light microscopy. In addition, the mild calcium treatment (1–10 mM, pH 6.0) has no effect on protoplast viability and regeneration. These factors make this an excellent model system for studying the dynamics of the fusion of natural membranes.

The first morphological response of protoplasts to 10 mM calcium as visualized by video microscopy was crenation or shrinkage of protoplasts [2,3]. When the crenated protoplasts adhered and fused, it took about 20 min to form a round fusion product. If the crenated protoplasts did not adhere to each other before re-expansion, fusion never occurred. These observations suggested that the exogenously added calcium was generating an osmotic driving force which was necessary for fusion to occur.

Several investigators have proposed that osmotic gradients can induce fusion of fusion permissive lipid bilayers [4–6], and it has been suggested that calcium may play a role in generating an osmotic gradient during chromaffin granule fusion [7]. If calcium enhances fusion of fusogenic protoplasts by inducing an osmotic gradient, then applying an osmotic gradient alone should

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enhance fusion, and applying an osmotic gradient in the absence of exogenous calcium should be sufficient to induce fusion. In this paper, fluorescence, video, and electron microscopy are used to monitor the effects of calcium and osmotic stress on protoplast fusion and subsequent mixing of the cytoplasms of the fusion products.

## Materials and Methods

*Stock cells cultures.* The methods for obtaining embryogenic suspension cultures and fusogenic cultures of wild carrot have been published elsewhere [1]. Briefly, suspension cultures of wild carrot were maintained in wild-carrot medium through biweekly subcultures. Aliquots of embryogenic carrot culture were transferred to fusion-inducing medium and weekly serial subcultures were made to obtain fusogenic cultures.

*Protoplast isolation.* Protoplasts were isolated from cells in fusion-inducing medium 4 days after transfer. Cells were collected on Whatman No. 1 paper and were incubated in a solution of 2% (w/v) Driselase (Plenum Sci. Lot No. KY 115), 0.4 molal sorbitol, 2.5 mM EGTA, and 1 mM Mes at pH 4.8. The cell-enzyme solution was placed on a rotary shaker (125 rpm; 25°C). After 2 h incubation, the protoplasts were filtered through a 41 $\mu$m mesh stainless steel screen to remove any clumps of unreleased cells and debris. The filtered protoplasts were centrifuged at approximately 40 $\times g$ for 5 min.

*Protoplast fusion.* Protoplasts were washed twice in an osmoticum of 0.45 molal sorbitol and used for fusion studies by the following protocols. In the first protocol, the normal calcium regime, a solution of 0.45 molal sorbitol containing 10 mM $CaCl_2$ was added. In the second protocol, the high osmotic stress regime, protoplasts were resuspended in 0.6 molal sorbitol for 10 min and a solution of 0.45 molal sorbitol plus or minus 10 mM $CaCl_2$ was added. In the third protocol, protoplasts which had been in 0.45 molal sorbitol were resuspended in 0.35 molal sorbitol plus or minus 10 mM $CaCl_2$. Protoplasts in the different osmotic regimes were fixed for electron microscopic studies as described below. For rapid light microscopic observations, protoplasts were placed on slide, covered with a cover slip, and the different osmotic solutions were perfused across the slide.

*Fluorescent labeling.* Various fluorescent dyes (carboxyfluorescein, Rhodamine 123, and Rhodamine B ethyl ester) were used to monitor mixing of cytoplasm during fusion. The fluorescent labeling of protoplasts was carried out as described previously [8].

*Fluorescence microscopy and video image.* The microscopic system consists of a Zeiss inverted microscope IM 35 equipped with neofluar 63X lens for observing differential interference contrast (DIC), and epifluorescence. The light source for fluorescence microscopy was a HBO 50W high-pressure mercury lamp used with Zeiss filter combination of exciters, chromatic beam splitters and barrier filters. For fluorescein, the filter combinations were exciter filter BP 450, beam splitter FT 510, and barrier filter BP 520–560. For rhodamine, the filter combinations were exciter filter BP 510–560, beam splitter FT 580, and barrier filter LP 590. The microscope was coupled to a Panasonic WV-1554 TV camera, and a Sony U-matic video cassette recorder with a Sony Trinitron television monitor. Photographs were made with a camera coupled to the reflex camera housing (CONTAX) with Kodak Ektachrome ASA 400 film and developed with Kodak E-6 processing. Photographs of the video image were obtained with Kodak technical pan film 2415 Estar-AH base and developed with full strength Diafine developer.

*Electron microscopy.* Protoplast fixation was carried out at 4°C and all solution changes were facilitated by centrifugation. All fixative and buffer solutions were osmotically balanced to minimize volume changes as determined by light microscopic observations. Protoplasts in 0.6 molal sorbitol with and without calcium were fixed in 2.5% glutaraldehyde in 0.2 molal sorbitol and 0.2 M sodium phosphate buffer (pH 7.2) for 2 h. Protoplasts in 0.45 molal sorbitol were prefixed in 0.25% glutaraldehyde in 0.2 molal sorbitol and 0.1 M sodium phosphate buffer (pH 7.2) for 5 min. Fixation was continued in 2.5% glutaraldehyde (same buffer) for 2 h. All treated protoplasts were washed in 0.05 M sodium phosphate buffer with three changes and then postfixed in 1% osmium tetroxide (same buffer) for 1 h. Fixed protoplasts

were dehydrated in a graded ethanol series by 10% increments and embedded in Spurr's epoxy resin [9]. Sections were mounted on uncoated grids and counterstained with uranyl acetate followed by lead citrate post-stain. Specimens were examined with a JEOL 100S TEM operated at 80 kV.

## Results

In order to determine the effects of osmotic stress on fusion, two different osmotic regimes were used. In the first, a high osmotic stress regime, when protoplasts were exposed to 0.6 molal sorbitol for 10 min, the volume of the protoplasts decreased immediately indicating that protoplasts were responding to the hypertonic solution. When the solution of 0.6 molal sorbitol was made relatively hypotonic by perfusing with 0.45 molal sorbitol, the protoplasts began to swell. Protoplasts which had been in close contact in 0.6 molal sorbitol appeared to fuse as they began to swell in response to the hypotonic solution. As shown in Fig. 1, fusion occurred and a spherical fusion product was formed within 30 s. Lowering the osmotic potential either with or without exogenous calcium was sufficient to cause fusion to occur.

With the light microscope it was difficult to discern whether membrane mixing might have occurred under the high osmotic stress regime when cytosolic mixing would be impaired. Thus, to delineate the requirements for calcium and osmotic stress, electron microscopic studies were conducted on protoplasts exposed to several different osmotic treatments with and without exogenously added calcium.

The plasma membrane of protoplasts in either 0.6 or 0.45 molal sorbitol without calcium frequently showed small areas of close proximity between protoplasts (Fig. 2a, b); whereas, the plasma membrane of protoplasts in 0.6 molal sorbitol with 10 mM calcium showed large planar areas of close apposition (Fig. 2c). Whether or not calcium was present, when the protoplasts were in 0.6 molal sorbitol, their plasma membranes were observed to be in close apposition but the bilayers had not fused.

Many of the protoplasts which had adhered in

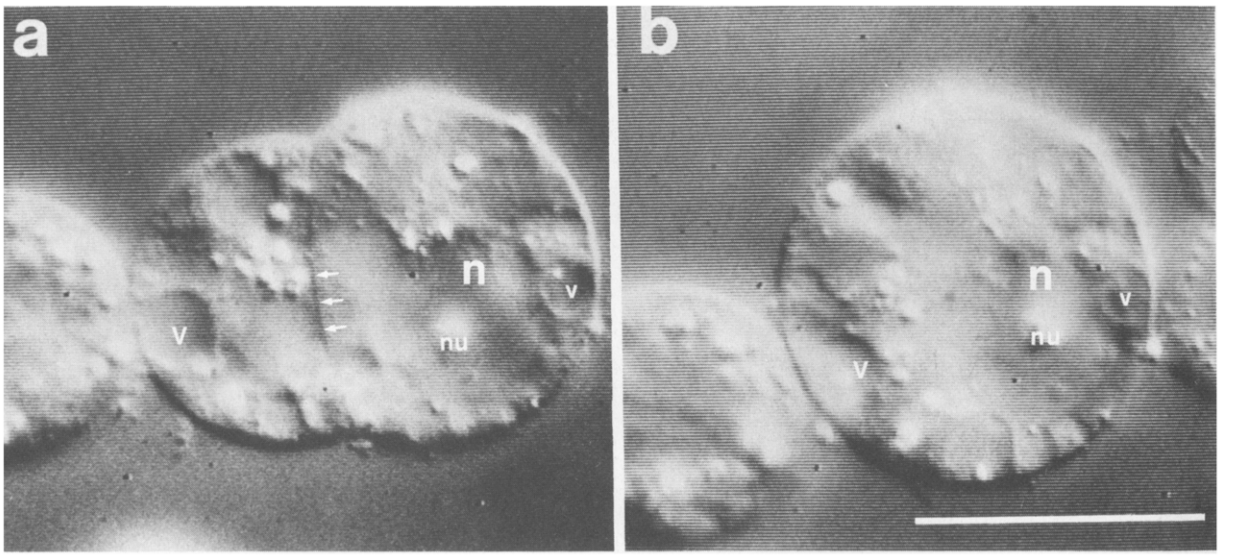



Fig. 1. Fusogenic carrot protoplasts were suspended in 0.6 molal sorbitol and fused by 10 mM calcium in 0.45 molal sorbitol. (a) As the calcium solution was perfused across the slide, the plasma membrane of two closely apposed protoplasts fused immediately. The osmotic swelling resulting from the lower osmolality caused this fast fusion but the fusion was not complete as a line (arrows) separating two cytoplasms was evident. (b) After a round fusion product was formed, the positions of organelles in this sphere were not much different from the parental protoplasts suggesting that the mixing of cytoplasms was slow and not complete even though the line separating the cytoplasms disappeared. The actual time sequence from (a) to (b) is 29 s. (n = nucleus, nu = nucleolus, v = vacuole). Bar = 20 $\mu$m.

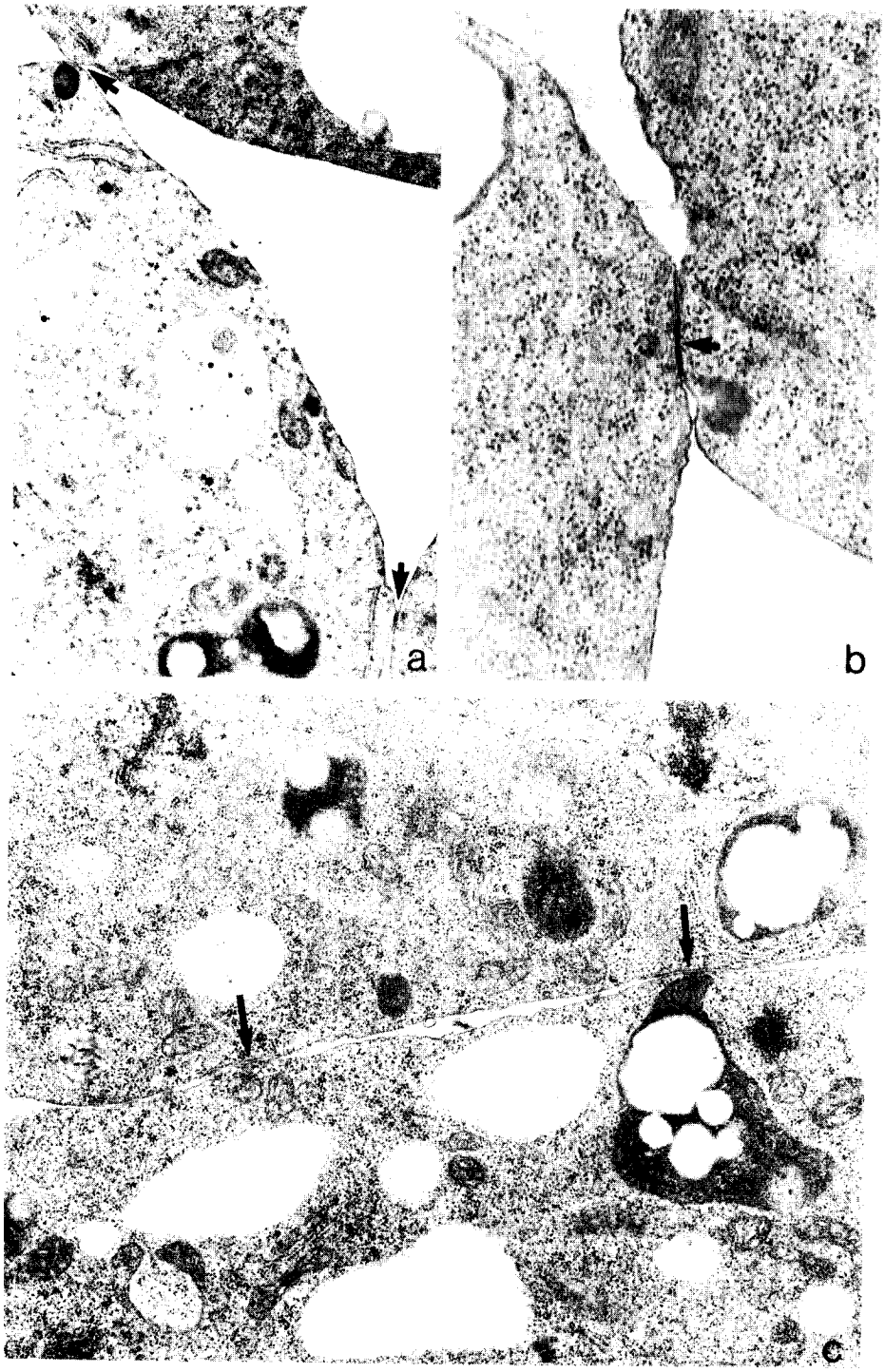



Fig. 2. Fusogenic protoplasts in different osmotic manipulations: (a) in 0.45 molal sorbitol without calcium, (b) in 0.6 molal sorbitol without calcium, and (c) in 0.6 molal sorbitol with 10 mM calcium. In all cases, protoplast plasma membranes were in close apposition (arrows) and no bilayer fusion was observed. (Magnification: a, ×8400; b, ×28000; c, ×14000).

0.6 molal sorbitol plus or minus calcium underwent fusion following the addition of 0.45 molal sorbitol either with or without calcium. Thin sections through the regions of contact between adhering protoplasts suggested that the membrane fusion of carrot protoplasts involved formation of a single, unit membrane intermediate (Fig. 3a, b). Sampling of many sections of protoplasts fixed at different time intervals during fusion always indicated a single site of membrane fusion and formation of a cytoplasmic connection bridge between the fusing protoplasts (Fig. 4a, b). These observations corroborated those seen with video microscopy, which suggested that membrane fusion occurred in the hypotonic solution, that exogenous calcium was not required, and that a single cytoplasmic connection formed.

While membrane fusion was rapid with the high osmotic stress regime, cytoplasmic mixing as observed with video and light microscopy was retarded (figure not shown). Fluorescent dyes also were used to monitor cytoplasmic mixing (Fig. 5).

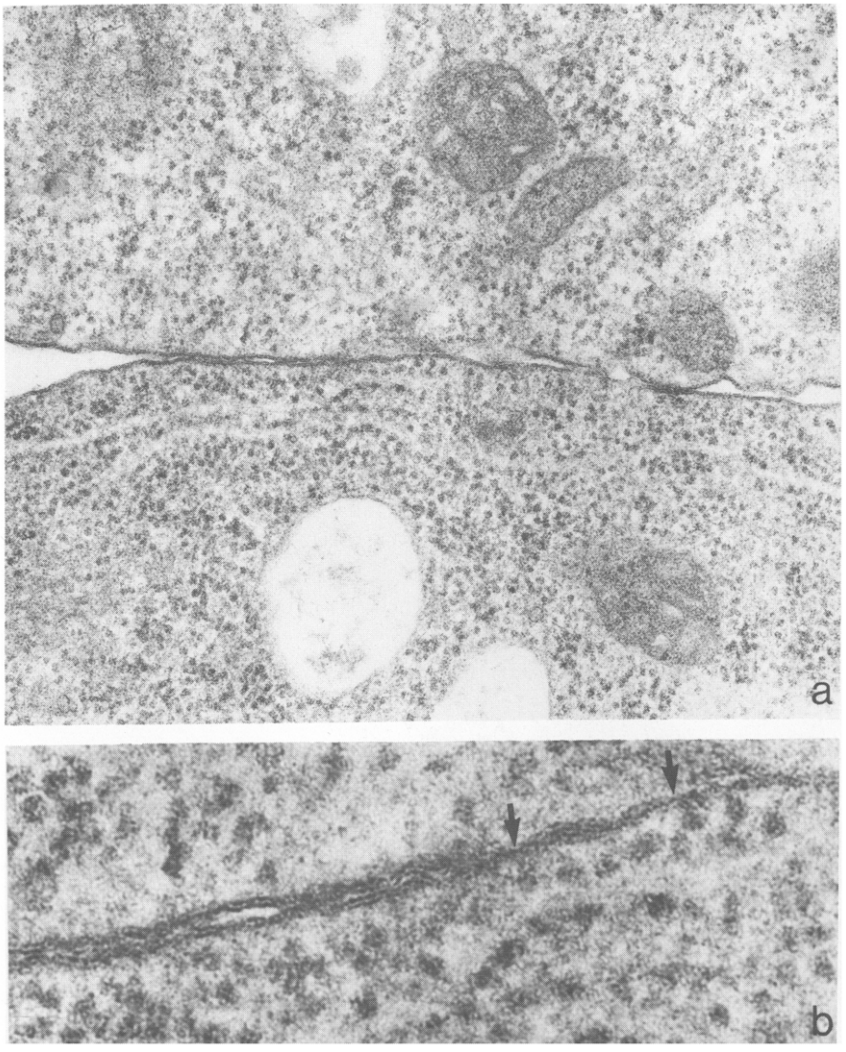



Fig. 3. Transmission electron micrographs of (a) fusogenic protoplasts in 0.6 molal sorbitol followed by 0.45 molal sorbitol containing 10 mM calcium. (b) Thin section through the region of contact between adhering protoplast membranes showed the single membrane intermediate (arrows). (Magnification: a, ×87000; b, ×290000).

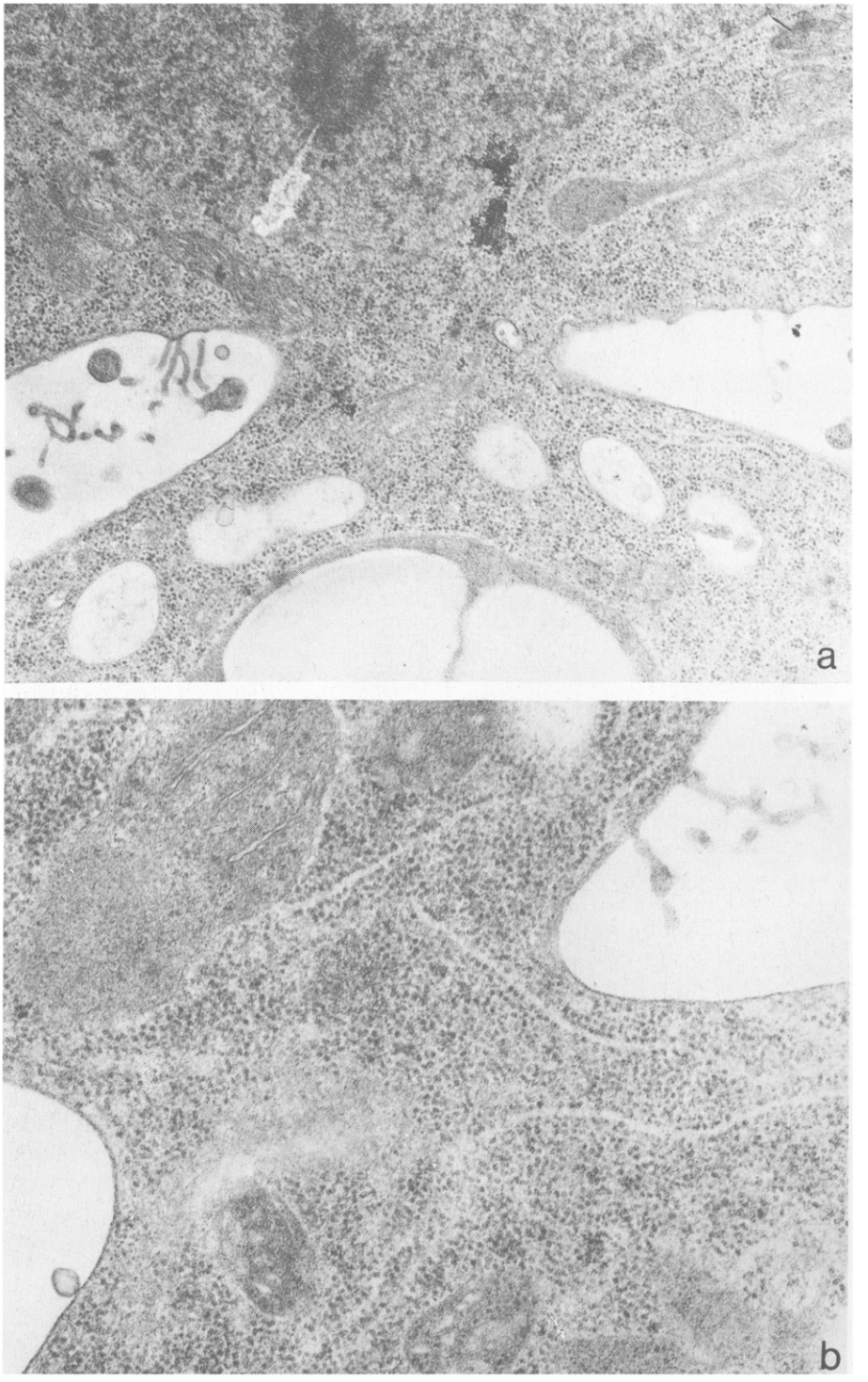



Fig. 4. Protoplasts fusing in 0.45 molal sorbitol (a, without 10 mM $CaCl_2$; b, with 10 mM $CaCl_2$). Intermediate stages during the formation of spherical fusion product indicate a single site of membrane fusion and formation of a cytoplasmic bridge. (Magnification: a, ×21 000; b, ×35 000).

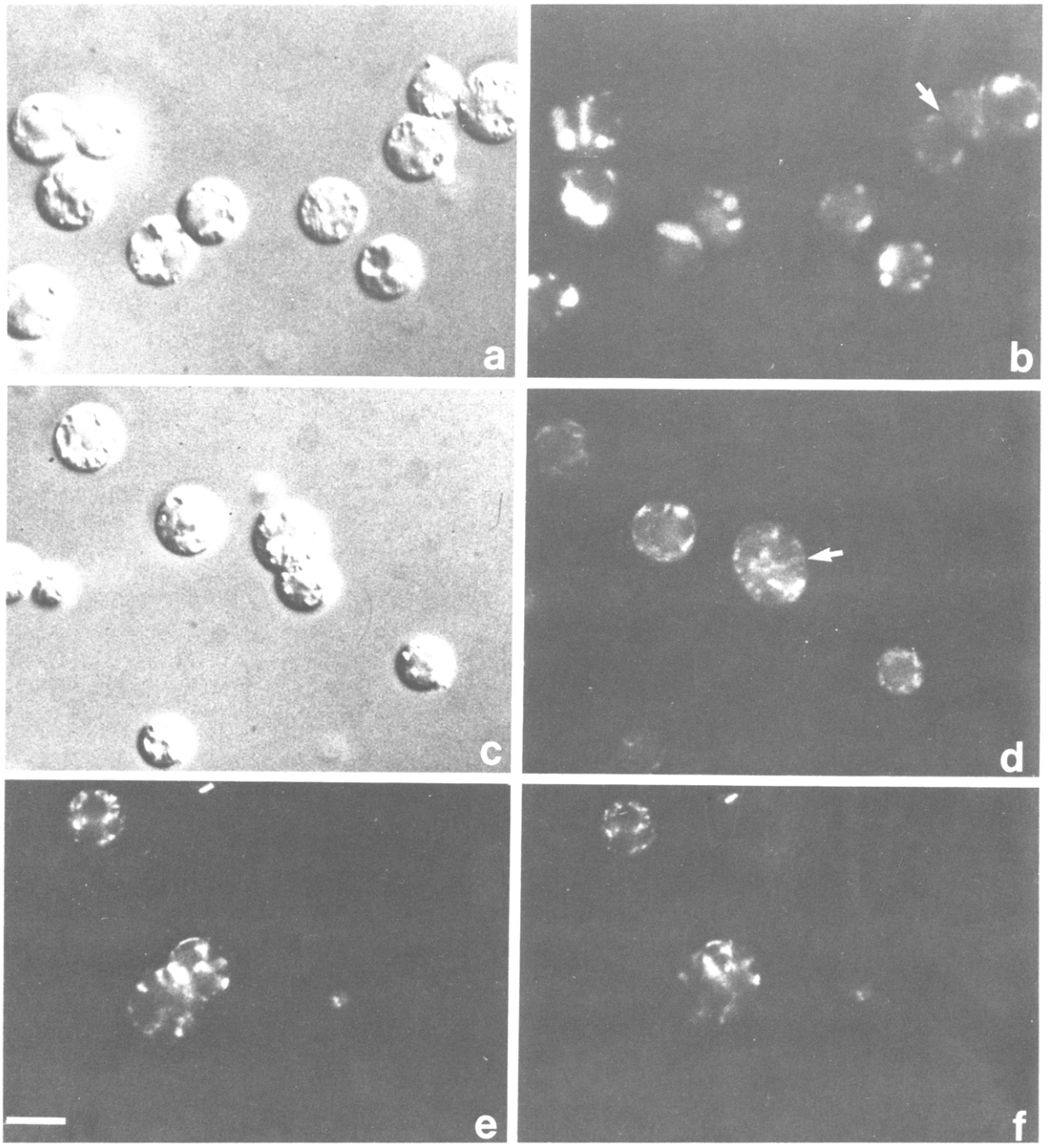
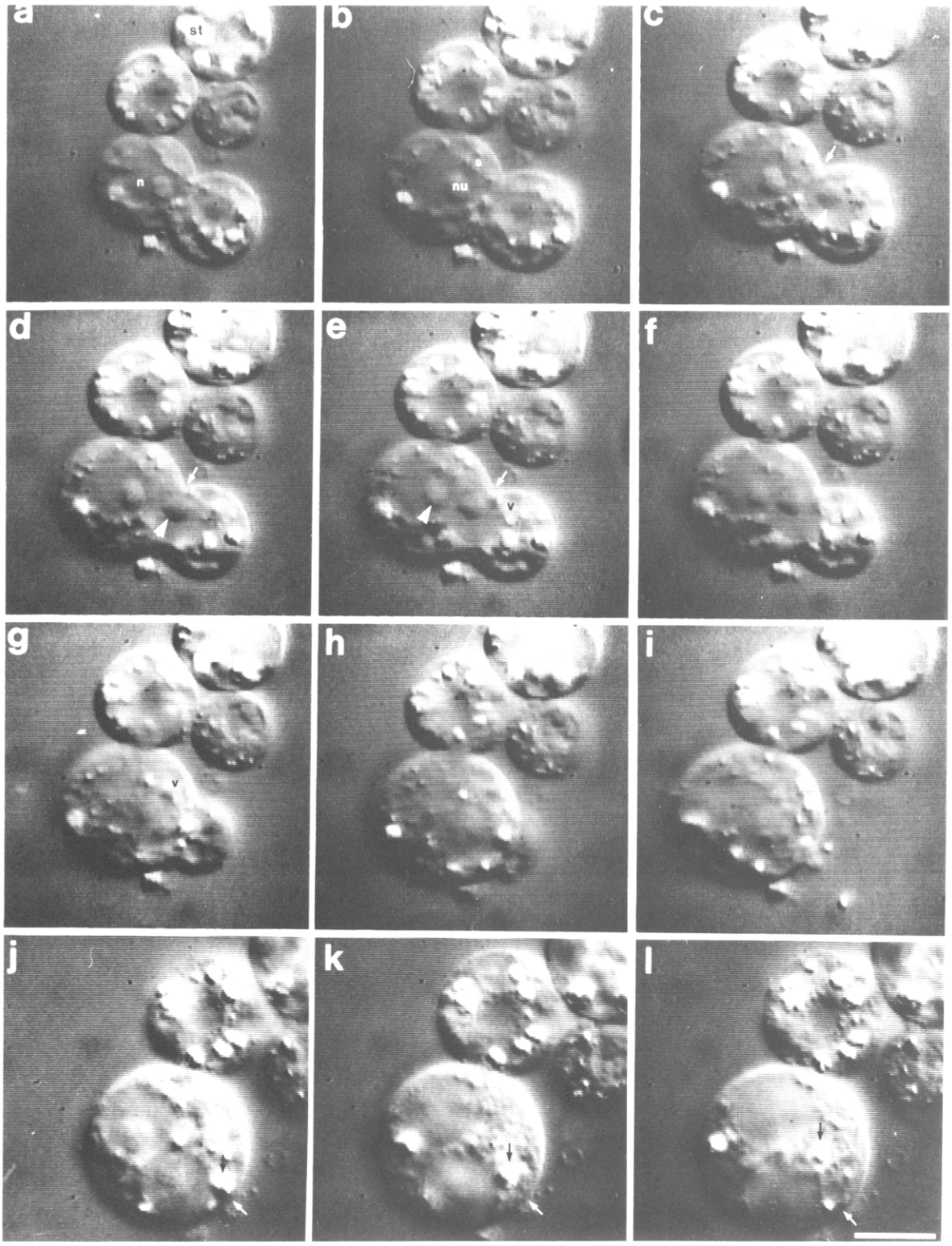



Fig. 5. Fusogenic carrot protoplasts labeled with different fluorescent dyes. The labeled protoplasts were plasmolyzed in 0.6 molal sorbitol and induced to fuse by bleeding across the slide a solution of 10 mM calcium in 0.45 molal sorbitol. (a) Carboxyfluorescein-labeled protoplasts just prior to fusion (DIC optics). (b) Same as (a), a few seconds later. Arrows indicate fusion products in epi-fluorescence, note localization of the labels in the vacuoles. (c) Freshly isolated protoplasts labeled with Rhodamine 123 for 30 min (DIC optics). (d) Same as (c), a few seconds later. Arrow indicates fusion product in epifluorescence, green fluorescence was exhibited using 485 nm excitation. (e) Protoplasts labeled with Rhodamine B ethyl ester for 30 min after isolation. Red fluorescence was emitted using 546 nm excitation. (f) The same protoplast as in (e) showing the final stage of the fusion process. Bar = 20 $\mu$m.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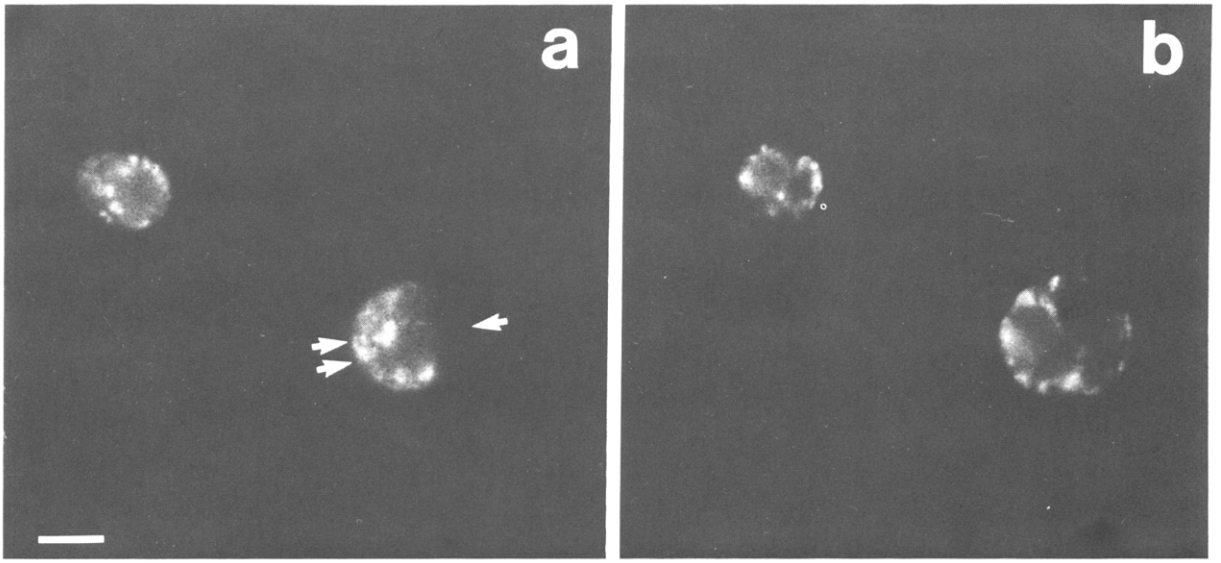


Fig. 7. Corresponding fluorescent micrographs of fusogenic protoplasts suspended in 0.45 molal sorbitol and fused by 10 mM calcium in 0.35 molal sorbitol. (a) Rhodamine 123-labeled protoplast (double arrows) and non-labeled protoplasts (arrow, not clearly seen) fusing. (b) After fusion was completely, labeled mitochondria were observed throughout the fusion product with 546 nm excitation. Bar = 20 $\mu$m.

When the vacuoles were labeled with carboxyfluorescein, it was evident that even though the fusing protoplasts were beginning to round out the vacuoles had not mixed. A non-fluorescent region appeared to separate the cytoplasm of the fusing protoplasts in Figs. 5b, d, and f. The slow mixing of the cytoplasm may have been a consequence of the decrease in cytoplasmic streaming observed at the higher osmoticum. As the osmoticum was lowered, increased saltatory motion was observed; however, the recovery of normal streaming and cytosolic mixing was delayed.

In the second or low osmotic regime, protoplast fusion was enhanced by lowering the osmotic potential from 0.45 molal sorbitol to 0.35 molal sorbitol with or without calcium. Fusion of protoplasts with this osmotic regime seemed to be slower than with the higher osmotic stress regime, but cytoplasmic mixing was faster. The time course of protoplast fusion with 10 mM calcium in 0.35 molal sorbitol was monitored with video microscopy and is shown in Fig. 6. Two protoplasts came into close contact with each other (Fig. 6a). The protoplast membrane fused, and a connection was formed between the two cytoplasms (Fig. 6 b–c). The nucleus, nucleolus, and other organelles could be seen. The cytoplasmic connection expanded and the cytoplasm of one protoplast appeared to move through this connection into that of the other protoplast. Migration of the nucleus, vacuoles, spherosomes, and starch granules was observed (Fig. 6 d–h). After 140 s the two proto-

Fig. 6. Time-course video images of fusogenic carrot protoplasts suspended in 0.45 molal sorbitol and induced to fuse by bleeding across the slide a solution of 10 mM calcium in 0.35 molal sorbitol. (a, b) A few seconds after initial contact of two protoplasts. Protoplast membrane became fused, and the cytoplasmic connection was formed. (c–h) The migration of the nucleolus, vacuoles, spherosomes and starch granules from one protoplast to another through the cytoplasmic connection. Movement of nucleolus (arrowhead) is shown from (c) to (e) relative to the stationary position of site of membrane fusing (arrow). (i) The spherical fusion product was formed. (j–l) The mixing of cytoplasms between both parental protoplasts were shown. Note the saltation of two nuclei from (j) to (l), also the localization of starch granule (black arrow) relative to plasma membrane margin (white arrow). The actual time sequence from (a) to (i) is 140 s and from (a) to (l) is 270 s. (n = nucleus, nu = nucleolus, s = spherosome, st = starch granule, v = vacuole). Bar = 20 $\mu$m.

plasts formed a spherical fusion product (Fig. 6i). Note that intermingling of the organelles of the two protoplasts was evident even before rounding. This was in contrast to the higher osmotic stress regime (Figs. 1 and 5). There was continued movement and apparent mixing of cytoplasm between both parental protoplasts after completion of the process (Fig. 6 j–l). Parallel experiments fusing Rhodamine 123-labeled and nonlabeled protoplasts with this lower osmotic stress regime confirmed the rapid mixing of the mitochondrial population within the fusion product (Fig. 7 a, b).

## Discussion

Osmotic gradients have been shown to be the driving force for bilayer fusion in artificial systems. Cohen et al. [4] and Akabas et al. [5] showed that with phospholipid vesicles made from either negatively charged lipids neutralized by calcium or uncharged lipids, calcium promoted the close association of these vesicles to the planar membrane and that fusion occurred only after an osmotic force was applied to induce vesicle swelling. In other fusion systems, Zimmerberg and Whitaker [7] demonstrated that the fusion of cortical secretory granules to the plasma membrane was prevented when the osmolality of the medium surrounding the sea-urching eggs was raised. When the external osmolality was lowered, the secretory granules fused with the plasma membrane.

Osmotic forces also have been shown to be important for fusion in plant protoplasts. As early as 1972 it was suggested that deplasmolyzing osmotic shock would enhance the formation of interspecific hybrids [10]. With PEG-induced fusion, while protoplasts adhered in the presence of PEG, very few fusions occurred as observed by light microscopy until PEG was diluted and osmolality lowered [11,12]. This would indicate that osmotic changes upon dilution of PEG caused fusion to occur; however, the PEG-induced plasmolysis would have inhibited cytoplasmic streaming and cytoplasmic mixing [12]. Therefore, fusion might have occurred prior to lowering the osmoticum, and protoplast fusion as observed at the light microscopic level would not be observed until the PEG was removed and cytoplasmic mixing could occur. Studies of erythrocytes, however, suggest that membrane fusion occurred only after dilution of PEG [13,14], and that osmotic stress was necessary for the fusion event.

With the fusogenic protoplasts, ultrastructural studies revealed that fusion did not occur under hypertonic conditions even when 10 mM calcium was added. Adding calcium increased the amount of membrane adhesion as seen at the electron microscopic level and protoplast aggregation seen at the light microscopic level. This would be expected since one effect of calcium would be to reduce the surface charge or repulsive interactions of the protoplast plasma membrane; however, the fact that fusion occurred when the protoplasts were placed in a relatively hypotonic solution (from 0.6 to 0.45 molal sorbitol) whether or not calcium was present, suggested that while exogenous calcium enhanced adhesion and fusion, it was not necessary to add exogenous calcium for fusion to occur.

While the mechanism by which osmotic stress enhances fusion is unknown, Fisher and Parker [6] suggested that first the aqueous solution between two bilayers must be removed to allow close contact and adhesion. Then as water flows into the fusing bodies causing swelling, the force of swelling would cause the lipid bilayers to mix. This senario is implied by our observations of the fusogenic carrot protoplasts. Increasing the osmolality of the external solution to 0.6 molal sorbitol should have promoted removal of the aqueous solution between the protoplasts at the contact site, and thereby enabling close apposition and adhesion. Lowering the osmolality of the sorbitol caused the protoplasts to swell and the resultant swelling served as the driving force for mixing of the adhering bilayers.

With a living system, discerning the precise stimulus for fusion resulting from osmotic stress is more difficult than with liposomes since changes in cellular physiology as a result of the stress may enhance fusion. For instance, it is possible that osmotic stress might have caused an increase in cytosolic calcium [15]. Previous studies have shown that increasing cytosolic calcium by adding A23187 in the absence of exogenous calcium was sufficient to enhance fusion of the fusogenic carrot protoplasts [16].

Osmotic stress also has been shown to affect

the synthesis of polyamines in plants. Flores and Galston [17] showed that the putrescine content of oat leaf cells and protoplasts increased up to 60-fold within 6 h of exposure to osmotic stress (0.4 to 0.6 molar sorbitol) and increased arginine decarboxylase activity paralleled the rise in putrescine. Polyamines have been implicated in membrane fusion [18,19], and inhibiting polyamine biosynthesis with α-dimethylfluoroarginine decreases the fusion potential of the fusogenic protoplasts [20]. Thus, an increase in polyamine biosynthesis induced by osmotic stress would be expected to favor protoplast fusion.

Although the hypertonic solution may have increased the levels of putrescine and intracellular calcium in fusogenic protoplasts, it is important to note that fusion did not occur under hypertonic conditions. Only when protoplasts were returned to a hypotonic solution was fusion observed. These observations are consistent with those using fusion permissive vesicles [4–7]. Furthermore, Creutz and Pollard [21] have proposed that exocytosis of chromaffin granules during adrenal medulla secretion, involves an increase in free calcium concentration within the cell which may provide the chemiosmotic driving force for membrane fusion. The fact that under isotonic conditions, external calcium causes transient crenation prior to fusion of fusogenic protoplasts [3] and that osmotic stress without the addition of external calcium will cause protoplast fusion supports this hypothesis.

In summary, we conclude that fusion of fusogenic carrot protoplasts can be caused by osmotic-induced swelling. While exogenous calcium enhances fusion and may contribute to the osmotic driving force under isotonic conditions, exogenous calcium is not required for fusion in this system. There appears to be only one site of fusion. This is based on data presented here and previous studies [3] where protoplasts were fused in a solution of fluorescent dextran (mol. wt. 20000). In no instance, were inclusions of exogenous solutions evident. In addition, fusion appears to involve the formation of a single, unit membrane. Finally, while the higher osmotic stress regime favors rapid membrane fusion, the milder of lower osmotic stress regime favors cytoplasmic mixing.

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## References

1 Boss, W.F., Grimes, H.D. and Brightman, A.O. (1984) Protoplasma 120, 209–215
2 Boss, W.F., Allen, N.S. and Grimes, H.D. (1983) 6th International Protoplast Symposium, Basel, Switzerland, Poster Proceedings, pp. 96–97
3 Boss, W.F. and Grimes, H.D. (1985) Beltsville Symposia on Frontiers of Membrane Research in Agriculture, Vol. 9, pp. 133–145
4 Cohen, F.S., Akabas, M.H. and Finkelstein, A. (1982) Science 217, 458–460
5 Akabas, M.H., Cohen, E.S. and Finkelstein, A. (1984) J. Cell Biol. 98, 1063–1071
6 Fisher, L.R. and Parker, N.S. (1984) Biophys. J. 46, 253–258
7 Zimmerberg, J. and Whitaker, M. (1985) Nature 315, 581–584
8 Kanchanapoom, K. and Boss, W.F. (1986) Plant Cell Reports (in press)
9 Spurr, A.R. (1969) J. Ultrastruct. Res. 26, 31–43
10 Keller, W.A., Harvey, B.L., Kao, K.N., Miller, R.A. and Gamborg, O.L. (1972) Coll. Inter. C.N.R.S. 212, 455–463
11 Kao, K.N. and Michayluk, M.R. (1974) Planta 115, 355–367
12 Wallin, A., Glimelius, K. and Eriksson, T. (1974) Planta 115, 355–367
13 Knutton, S. (1979) J. Cell Sci. 36, 61–72
14 Hui, S.W., Isac, T., Boni, L.T. and Sen, A. (1985) J. Membrane Biol. 84, 137–146
15 Grinstein, S., Rothstein, A. and Cohen, S. (1985) J. Gen. Physiol. 85, 765–787
16 Grimes, H.D. and Boss, W.F. (1985) Plant Physiol. 79, 253–258
17 Flores, H.E. and Galston, A.W. (1982) Science 217, 1259–1260
18 Hong, K., Schuber, F. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 732, 469–472
19 Schuber, F., Hong, K., Düzgüneş, N. and Papahadjopoulos, D. (1983) Biochemistry 22, 6134–6140
20 Grimes, H.D., Slocum, R.D. and Boss, W.F. (1986) Biochim. Biophys. Acta 886, 130–134
21 Creutz, C.E. and Pollard, H.B. (1983) J. Auton. Nerv. Syst. 7, 13–18