Characterization of the pH-Induced Fusion of Liposomes with the Plasma Membrane of Rye Protoplasts†

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ABSTRACT: We present evidence that at acidic pH, liposomes composed of soybean lipids fuse with the plasma membrane of protoplasts isolated from rye leaves (*Secale cereale* L. cv Puma). Using the resonance energy transfer assay (RET), we determined the rate and extent of liposome and protoplast plasma membrane lipid mixing. The fluorescent donor–acceptor pair was *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE). Fusion was substantial below pH 5, and the half-time of lipid mixing was fast ($t_{1/2}$ on the order of minutes) and pH, concentration, and temperature dependent. The extent of liposome and protoplast fusion from the total amount of liposomes associated with the protoplasts was also determined by the RET assay. Protoplasts were incubated with fluorescent-labeled liposomes (5 min at 30 °C) at different pH values and then washed twice by centrifugation. The fluorescence spectra of the protoplast suspension permitted determination of the ratio of N-NBD-PE emission at 530 nm to the N-Rh-PE emission at 590 nm, which is a measure of the degree of lipid mixing. Addition of 2% (v/v) Triton X-100 to these suspensions permitted determination of the total amount of N-NBD-PE associated with the protoplasts. The amount of liposomes associated (fused and unfused) with protoplasts at pH 3.9 was approximately 9 times greater than that at pH 5.6. Approximately 64% of the liposomes associated with protoplasts were fused with the protoplasts at pH 3.9, and only 9% at pH 5.6. The transfer of liposome contents to the protoplast interior was studied with a method based on the fluorescence enhancement of a solution of calcein, initially confined in the liposomes at self-quenching concentrations. The kinetics of calcein release were very similar to those of lipid mixing. Fluorescence microscopy showed that after fusion with liposomes containing calcein, the protoplasts exhibited a strong diffuse fluorescence in the interior. Further evidence that the enhancement in calcein fluorescence was not due to release of the dye in the aqueous media came from experiments employing protoplast pellets. After fusion of protoplasts with calcein-containing liposomes, the protoplasts were washed by two centrifugations. Fluorescence intensity measurements and fluorescence microscopy observations showed that protoplasts in the pellets retained a strong calcein fluorescence.

Liposome interactions with biological membranes have been widely investigated in the last decade; however, the majority of these studies have focused on membranes of mammalian cells and less on plant cells. Interest in plant cell interaction with liposomes has emerged from the possibility of introducing exogenous macromolecules into the cytoplasm. Thus, genetic material such as RNA and DNA encapsulated into liposomes was delivered to plant protoplasts after incubation of protoplasts with liposomes in the presence of poly(ethylene glycol) or poly(vinyl alcohol) (Fernandez et al., 1978; Rollo et al., 1981; Matthews & Cress, 1981; Fukunaga et al., 1981; Nagata et al., 1981; Fraley et al., 1982; Freeman et al., 1984). However, in the presence of these "fusogens", liposomes enter the protoplasts via endocytosis (Fukunaga et al., 1983; Nagata, 1984). The endocytotic pathway was proposed as the most probable mechanism of transfer of liposome contents to protoplasts in a system employing pH-sensitive liposomes and poly(ethylene glycol) (Wang et al., 1986).

Our interest in protoplast–liposome fusion lies in the possibilities of altering the plasma membrane lipid composition of the protoplasts by fusion with liposomes. When cold-hardy plant species, such as winter cereals, are exposed to low, above-zero temperatures, they become cold acclimated such that their tolerance of freezing temperatures is increased. The cold acclimation process dramatically alters the cryobehavior of the plasma membrane (Steponkus, 1984). For example, freeze-induced osmotic contraction results in endocytotic vesiculation of the plasma membrane of protoplasts isolated from nonacclimated (NA)[1] rye (*Secale cereale* L. cv Puma) leaves (Dowgert & Steponkus, 1984; Gordon-Kamm & Steponkus, 1984a), whereas in protoplasts isolated from acclimated (ACC) leaves, it results in the formation of exocytotic extrusions (Gordon-Kamm & Steponkus, 1984b). In NA protoplasts, freeze-induced dehydration results in lateral phase separations and lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane, changes that, under similar conditions, are not observed in ACC protoplasts (Gordon-Kamm & Steponkus, 1984c; Pihakaski & Steponkus, 1987). At the molecular level, the cold acclimation process results in sub-

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[1] Abbreviations: ACC, cold acclimated; FITC-D150, fluorescein isothiocyanate labeled dextran of 150-kDa molecular mass; kDa, kilodalton(s); NA, nonacclimated; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PE, phosphatidylethanolamine; Rh, lissamine rhodamine B sulfonyl; RET, resonance energy transfer.

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stantial changes in the lipid composition of the plasma membrane (Lynch & Steponkus, 1987). We believe that these changes in the lipid composition are related to the altered cryobehavior of the protoplasts isolated from rye leaves. One way to establish a causal relationship between the difference in lipid composition and the changes in cryobehavior of the protoplasts is to alter the lipid composition of the plasma membrane by fusion with liposomes. Low pH has been used to effect liposome fusion with a wide range of biological membranes and cell types, e.g., mitochondrial inner membranes (Schneider et al., 1980), Semliki Forest virus (White & Helenius, 1980), Sendai virus (Amselem et al., 1986), influenza virus (Stegmann et al., 1985), cells that are infected with influenza virus (van Meer & Simon, 1983), membrane vesicles derived from *Bacillus subtilis* (Driessen et al., 1985), erythrocyte ghosts (Arvinte et al., 1986), and mouse liver nuclei (Arvinte et al., 1987a,b).

In the present study we show that in the range between pH 4 and 5 liposomes composed of soybean lipids fuse with the plasma membrane of protoplasts isolated from nonacclimated leaves of rye (*S. cereale* L. cv Puma). The fusion process was assayed by techniques that showed mixing of the liposome and protoplast membrane lipids and by transfer of the liposome contents to the protoplast interior.

Materials and Methods

Chemicals. Calcein [2′,7′-bis[[bis(carboxymethyl)-amino]methyl]fluorescein], fluorescein isothiocyanate–dextran of average M_r 156000 kDa (FITC-D150), and D-sorbitol were obtained from Sigma (St. Louis, MO).

Lipids. *N*-(7-Nitro-2-1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine were purchased from Avanti, Birmingham, AL. A mixture of soybean lipids containing approximately 40% L-α-phosphatidylcholine was obtained from Sigma (P 3644).

Liposome Preparation. For the resonance energy transfer assay, liposomes were prepared by sonication. Stock solutions of soybean lipids, N-NBD-PE, and N-Rh-PE in a molar ratio of 9.8:0.175:0.025 were mixed, and the solvent was evaporated under nitrogen. A 0.5-mL aliquot of a 0.527 osmolal (osm) sorbitol solution was added over the dried lipid film, and the liposomes were formed by sonication with a Branson 1200 bath sonicator. Sonication for 5–10 min was sufficient to obtain a clear solution (i.e., no change in turbidity or in the appearance of the liposomes was observed with further sonication).

Liposomes containing calcein were made from the same soybean lipids without N-NBD-PE and N-Rh-PE. The 0.5 mL of 0.527 osm sorbitol solution that was added over the dried lipid film contained 39 mg of calcein (2 drops of 6 N NaOH were needed to solubilize the calcein). After sonication, the unincorporated calcein was removed from the liposomes by filtration over five small Sephadex G-2000 columns (made from Pasteur glass pipets coated with Sigmacote from Sigma). The fractions containing the liposomes encapsulating calcein were mixed and used for fusion experiments with protoplasts within 3 h after preparation. Liposomes containing FITC-D150 were prepared in a similar way. The 0.527 osm sorbitol solution (0.5 mL) that was added over the lipid film contained 20 mg of FITC-D150. The sonication and column filtration steps were performed as described for liposomes containing calcein.

Plant Material and Protoplast Isolations. Seeds of *S. cereale* L. cv Puma were germinated and grown in vermiculite under a controlled environment (16-h light period at 20 °C and 8-h dark period at 15 °C). The plants used for protoplast isolation were between 10 and 15 days old (after the day of sowing). Protoplasts were enzymically isolated from leaves as previously described (Dowgert & Steponkus, 1984). After the enzymic digestion of the cell wall and washing by two centrifugations, the protoplasts were resuspended in isotonic sorbitol solution (0.527 osm). Protoplast concentrations in stock suspensions were determined in a hemocytometer.

Resonance Energy Transfer Measurements. The mixing of lipids from liposomes and protoplasts was monitored by the resonance energy transfer method (Struck et al., 1981; Hoekstra, 1982). Preliminary experiments were performed to determine the optimum ratio of soybean lipids, N-NBD-PE, and N-Rh-PE. The efficiency of resonance energy transfer, E, was calculated from donor quenching with the equation

$$E = 1 - F_1/F_0 \quad (1)$$

where the steady-state fluorescence intensity at 530 nm of NBD (energy donor) is measured in the presence (F_1) and in the absence (F_0) of the energy acceptor (Rh-chromophore) (Fung & Streyer, 1978; Struck et al., 1981). The relationship of transfer efficiency to acceptor probe density was tested by preparing different liposome suspensions in which the molar density of N-Rh-PE was varied from 0.0 to 1.0 mol % at a constant N-NBD-PE density of 1.75 mol %.

For RET measurements it is desirable to have initial concentrations of donor and acceptor molecules that give a large E value and for which small changes in fluorophore densities give measurable changes in E (i.e., to be in the range where the efficiency of energy transfer is nearly proportional to the ratio of N-Rh-PE to total lipid in the vesicle bilayer) (Struck et al., 1981). These conditions were fulfilled for soybean lipid liposomes having 1.75 mol % N-NBD-PE and 0.2 mol % N-Rh-PE ($E = 0.65$; the mole percent values were calculated by counting a mean molecular weight value of 700 for the soybean lipid mixture). Fluorescence measurements were performed on a Perkin-Elmer MPF-44B spectrofluorometer in a thermostated sample holder. To avoid artifacts due to settling of fluorescent materials, the suspension was continuously mixed during the measurements (the mixing must be gentle due to the fragility of the plant protoplasts). Studies of the lipid mixing by resonance energy transfer were performed by kinetic and steady-state measurements.

Kinetic Measurements of the Lipid Mixing. A typical assay mixture in the cuvette contained 3 mL of sodium acetate buffer [acid–base pair CH_3CO_2H/NaOH (0.02 N), 0.15 M NaCl, and sorbitol; final buffer osmolality being 0.5], 20 μL of liposome suspension (in the 0.527 osm sorbitol solution; 0.015 mM final lipid concentration), and 10 μL of a suspension of protoplasts (in isotonic 0.517 osm sorbitol; 8×10^4 protoplasts). The pH values indicated in the text were obtained in separate experiments from mixtures of the three solutions. In the concentration-dependent measurement, the amounts of liposomes and protoplasts were varied, but always in the same order or magnitude of the values presented previously. From temperature-dependent kinetic measurements the apparent activation energy for the lipid mixing (E_a) was obtained by the Arrhenius equation, $\ln K = \ln A - E_a/RT$, with the rate constant $K = \ln 2 / t_{1/2}$; $t_{1/2}$ is the half-time of the lipid mixing process measured from curves plotting the increase of NBD intensity in time, A is a constant, R is the universal gas constant, and T is the temperature in degrees Kelvin.

Steady-State Measurements of the Lipid Mixing. In this experiment, higher concentrations of liposomes and protoplasts were used. Thus, over the 3 mL of sodium acetate buffer were added 50 μL of liposome suspension (0.7 mM final lipid

concentration) and 100 μL of protoplast suspension (2×10^5 protoplasts). The incubation time at all pH values was 5 min at 32 °C. After being washed by two centrifugations (12 min each, ~120*g*) the pellets were resuspended in the same buffers and the emission spectra were registered (λ_{ex} = 475 nm). The relative NBD fluorescence intensity of each sample was also measured after incubating the samples for 5 min with 2% (v/v) Triton X-100 (under gentle shaking, conditions in which Triton X-100 solubilized the protoplast and liposome lipids). The solubilization of the lipids in the Triton X-100 micelles suppresses the resonance energy transfer from NBD to Rh that takes place in the lipid bilayer. (One main requirement for efficient energy transfer is a small distance between the energy donor and energy acceptor molecules, in the range of 50 Å.) These intensity values of Triton-treated samples are proportional to the amount of N-NBD-PE lipids associated with the protoplasts. They are thus a measure of the liposome protoplast interaction.

The percent of fused liposomes from the total amount of liposomes associated with the protoplasts was defined from the *R* values:

$$\% \text{ fusion} = \frac{(R_f - R_i) - 0.06}{R_T - R_i} \times 100 \quad (2)$$

where

R = fluorescence NBD (530 nm)/ fluorescence Rh (590 nm) (3)

and the indices f, i, and T stand for final, initial, and after adding of Triton X-100, respectively. The value 0.06 represents the correction due to filter effects of protoplasts. The ratio *R* was shown to be a sensitive measure of the efficiency of the resonance energy transfer between N-NBD-PE and N-Rh-PE (Struck et al., 1981) and was used to quantify the amount of fusion between liposomes (Struck et al., 1981; Connor et al., 1984).

Assays for Transfer of Liposome Contents to Protoplasts. Mixing of the liposome and protoplast aqueous contents was shown by using liposomes encapsulating calcein at high self-quenching concentrations (Allen & Cleland, 1980; Connor et al., 1984). Kinetic and steady-state measurements using the calcein assay were performed.

Kinetic Measurements of Contents Mixing. Liposomes (100 μL; ~0.7 mM lipids) encapsulating calcein at self-quenching concentrations were equilibrated in 3 mL of sodium acetate buffer to which a 200-μL protoplast suspension was added (5.4×10^5 protoplasts). From the increase in calcein fluorescence intensity (λ_{ex} = 490 nm; λ_{em} = 518 nm) monitored immediately after the addition of protoplasts (the initial decrease is due to protoplast filter effect), the pH dependence of the kinetics was obtained. Fluorescence microscopic observations showed that the protoplasts were fluorescent after the calcein steady-state fluorescence intensity reached equilibrium. To be sure that the increase in calcein fluorescence monitored after mixing of protoplasts with calcein-containing liposomes is the result of fusion and internalization of the calcein in the protoplasts rather than uptake from the surrounding medium, we performed the following steady-state measurements.

Steady-State Measurements of Contents Mixing. Liposomes encapsulating calcein (0.7 mM lipids) were incubated with protoplasts (1×10^5 protoplasts) in sodium acetate buffer (40 min, 30 °C, different pH's). After being washed by two centrifugations (1.2 min, 120*g*), the pellets were resuspended in 2 mL of the same buffer. The fluorescence intensities of these samples were measured before (F_c) and after (F_c^T) addition of 2% (v/v) Triton X-100. A measure of the percent

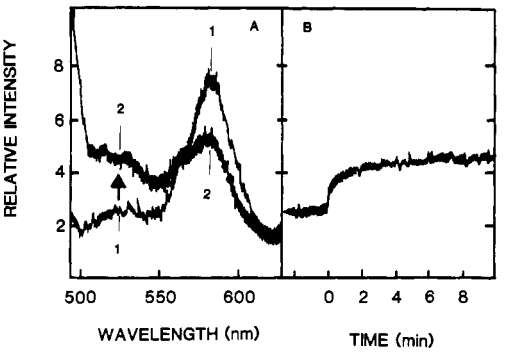

FIGURE 1: (A) Emission spectra before (curve 1) and after (curve 2) mixing of liposomes labeled with N-NBD-PE and N-Rh-PE with protoplasts (λ_{ex} = 475 nm, 23 °C, pH 3.94). Curve 2 represents the emission spectrum of the mixture at equilibrium (after 10 min). (B) Time dependence of the increase at 530 nm of NBD emission intensity after adding protoplasts to the liposome suspension (corresponds to the change from curve 1 to 2 in Figure 1A).

of fused liposomes can be determined from the increase in calcein fluorescence after reaction with Triton X-100 by using the equation

$$\% \text{ fusion} = (F_c - F_v)/F_c^T \times 100 \quad (4)$$

where F_v is the fluorescence intensity of calcein confined inside of the liposomes that are associated with the protoplast pellet. In the assumption that $F_v \ll F_c$, eq 4 becomes

$$\% \text{ fusion} = F_c/F_c^T \times 100 \quad (5)$$

Equation 5 is thus a measure of the percent of fused liposomes from the total amount of protoplast-associated liposomes, assuming that the fluorescence of the calcein confined in liposomes is negligible because of self-quenching concentrations (i.e., F_c originates mainly from free calcein inside the cytoplasm of protoplasts at nonquenching concentrations and not from the calcein contained in unfused liposomes).

Protoplast fluorescence after fusion with liposomes was observed with a Nikon Fluophot or a epi-fluorescence Universal Zeiss microscope. Osmolalities of the solution were measured with a Osmette A freezing point osmometer.

RESULTS

Lipid Mixing. The kinetics of mixing of liposome and protoplast lipids during fusion were measured by the RET method (Struck et al., 1981; Hoekstra, 1982). To use the RET method with plant protoplasts, special conditions must be chosen for diminishing the influence of chlorophyll absorption and fluorescence and to minimize filter effects. We solved these problems using small concentrations of protoplasts and liposomes (for details see Materials and Methods). In Figure 1A, curve 1 is a typical emission spectrum of a population of soybean liposomes labeled with 1.75 mol % N-NBD-PE and 0.2 mol % N-Rh-PE. From the recorded curves of the time evolution of the N-NBD-PE emission intensity after adding the protoplast suspension (Figure 1B), we determined fusion half-times and equilibrium emission intensities. Experiments similar to those presented in Figure 1 show that the fusion process is concentration, temperature, and pH dependent. Thus, the fusion kinetics were faster (smaller half-time values) with increasing liposome and/or protoplast concentration (Table I). A proportional increase in the concentration of liposomes and protoplasts does not change the percent of fusion but does reduce the half-time of the reaction (Table I). The fusion reaction occurs faster at low pH's: for pH 3.9, 4.5 and 4.9 the half-times of fusion of protoplasts with liposomes were 0.4, 2.2, and 3 min, respectively (for fusion conditions see Materials and Methods, t = 33 °C; errors were ±10% of the

Table I: Concentration Dependence of the Fusion Reaction[a]

liposomes (μg/mL)	protoplasts per mL (×10⁻⁴)	liposome lipids per 10⁶ proto-plasts[b] (μg)	liposomes fused[c] (%)	$t_{1/2}$[d] (min)
0.2	3.9	5.13	74	4.75 ± 0.5
0.4	3.9	10.26	30	2.5 ± 0.5
0.8	7.8	10.26	29	1.4 ± 0.4
1.6	15.6	10.26	30	1.2 ± 0.2
1.6	31.2	5.13	71	1.3 ± 0.2

[a] Liposomes containing N-NBD-PE and N-Rh-PE were incubated (pH 3.94, 25 °C) with rye protoplasts as described under Materials and Methods. [b] Values represent the ratio of the concentrations of liposomes to the concentration of protoplasts in the reaction media (calculated from the first two columns of the table). [c] Values for percent of liposomes fused with protoplasts after the reaction reached equilibrium represent the percent of fused liposomes from the total amount of liposomes initially present in the mixtures and were calculated from the increase in the R values (eq 2 and 3). [d] Half-time of lipid mixing during fusion, $t_{1/2}$, was measured from recorded curves of the time evolution of the N-NBD-PE emission intensity after adding protoplasts over a liposome suspension.

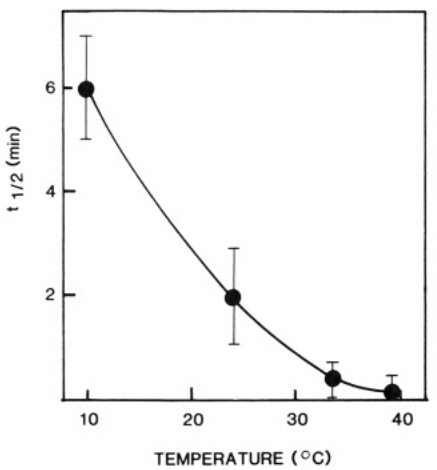

FIGURE 2: Temperature dependence of half-times of fusion of liposomes with rye protoplasts. The $t_{1/2}$ values were measured from the increase in time in NBD fluorescence after mixing protoplasts with liposomes (see Materials and Methods). All incubations were at pH 3.94.

values). The half-time of fusion decreases with an increase in temperature (Figure 2). From the temperature-dependence data, an apparent activation energy (E_a) of 21 ± 5 kcal/mol for the lipid mixing is obtained.

After incubation with protoplasts, liposomes can be associated with protoplasts in the following ways: (i) fusion; (ii) adhesion at the protoplast surface; (iii) internalization as a result of fusion (multilamellar liposomes) and/or liposome endocytosis. To determine the percent of liposomes fused with protoplasts from the total amount of liposomes associated with protoplasts, the following experiments were performed: Liposomes labeled with N-NBD-PE and N-Rh-PE were incubated with protoplasts at different pH values (Figure 3) (see Materials and Methods). After the fusion reaction was completed at different pH values, free liposomes in the medium were separated from the protoplast-associated liposomes by two centrifugations. (Control experiments have shown that under the conditions used for centrifugation, liposomes were not sedimented.) After resuspension in the corresponding buffer, the protoplast pellets were found to contain NBD- and Rh-labeled lipids (Figure 3). With decreasing pH, the Rh fluorescence maximum at 590 nm disappeared and an increase in intensity occurred in the region of the NBD fluorescence maximum (530 nm) (Figure 3). The fact that a strong lipo-

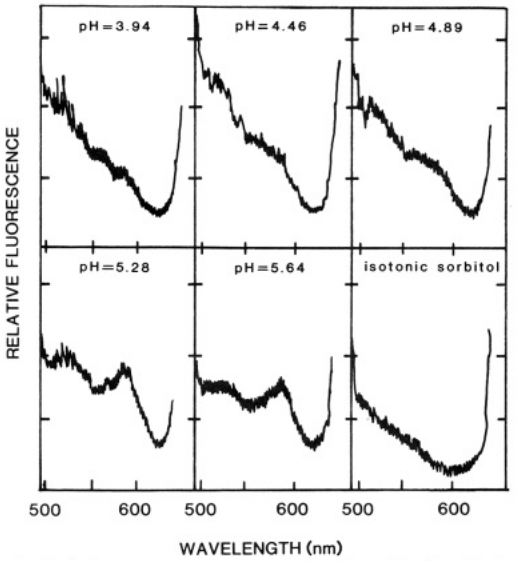

FIGURE 3: Emission spectra of protoplast pellets after incubation with liposomes at different pH's. Rye protoplasts were incubated with lipsomes containing N-NBD-PE and N-Rh-PE for 5 min at 32 °C. After being washed by two centrifugations, the protoplast pellets were resuspended in the corresponding buffer and the emission spectra were registered (λ_{ex} = 475 nm; see Materials and Methods). The pH values of the reaction mixtures are indicated; the spectrum index "isotonic sorbitol" corresponds to incubation of liposomes and protoplasts in 0.527 osm sorbitol. In all experiments the initial concentrations of protoplasts and liposomes were the same.

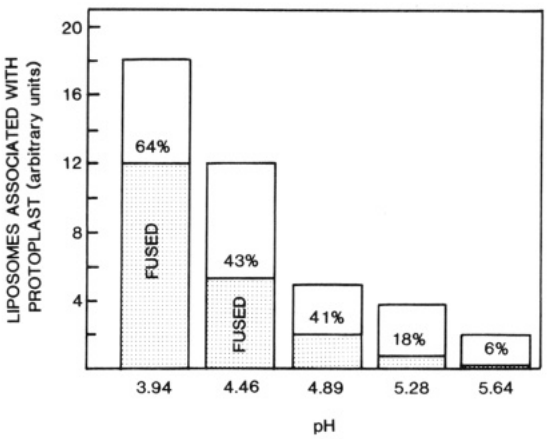

FIGURE 4: pH dependence of the amount of liposomes associated with protoplasts. After reaction of the samples shown in Figure 3 with 2% Triton X-100, the NBD fluorescence intensity was measured. The percent of fused liposomes (shaded rectangles) from the total amount of liposomes associated with the protoplasts was obtained as described in the text.

some–protoplast interaction occurred with decreasing pH was clearly shown after incubating the samples from Figure 3 with Triton X-100. The NBD fluorescence intensity of these Triton-treated samples (heights of the unshaded rectangles in Figure 3) are proportional to the amount of NBD-labeled lipids associated with the protoplasts and thus are also proportional to the number of liposomes associated with the protoplasts. By subtracting the strong light-scattering effect of the samples (the emission spectra corresponding to "isotonic sorbitol" in Figure 3) from the emission spectra at different pH's (Figure 3) we calculated R_f values (see eq 2). By use of eq 2 and R_i corresponding to the initial liposome suspension, the percentage of fused liposomes from the total amount of protoplast-associated liposomes was calculated (see Figure 4, the shaded rectangles).

Study of the Transfer of Liposome Contents to the Protoplast Interior. Mixing of the aqueous phases of liposomes

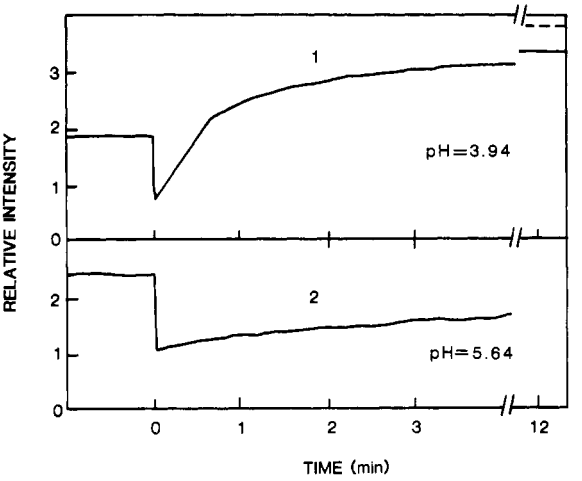

FIGURE 5: Kinetic measurements of the interaction between liposomes and protoplasts measured by the release of calcein from liposomes. Over a liposome suspension equilibrated at 34 °C for 4–5 min in the acidic buffer, protoplasts were added at time zero (see Materials and Methods). The increase in fluorescence intensity was measured at 518 nm (λ_{ex} = 490 nm). The pH's of the reaction media were 3.94 and 5.64 for curves 1 and 2, respectively. In the case of curve 1, equilibrium values (after 10 min) and the value of calcein fluorescence after adding 2% (v/v) Triton X-100 (- - -) are shown.

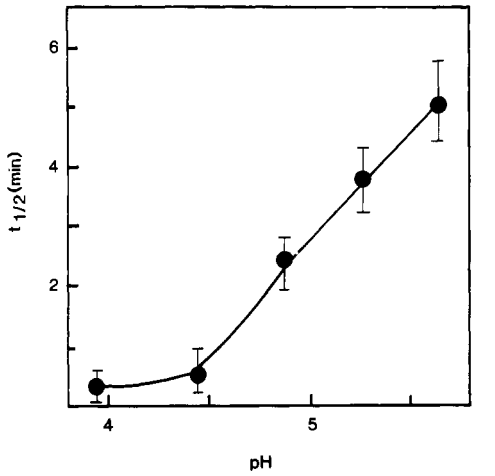

FIGURE 6: pH dependence of half-time values of calcein release ($t_{1/2}^c$) during the interaction between rye protoplasts and calcein-containing liposomes. The ($t_{1/2}^c$) values were measured from the increase in calcein fluorescence intensity with time.

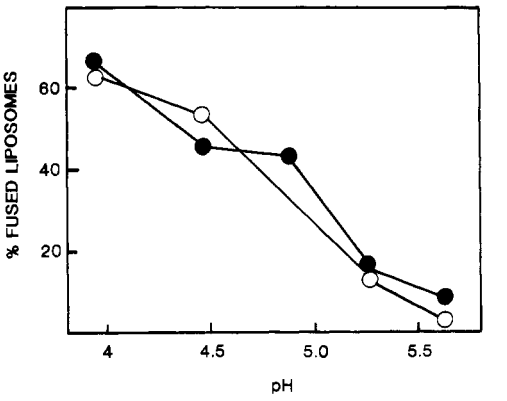

FIGURE 7: pH dependence of the percent of liposomes fused with protoplasts. Liposomes containing calcein at self-quenching concentrations were incubated with protoplasts as described under Materials and Methods. The percentage of fused liposomes was calculated by using eq 5 (○). For comparison, the percentage of fused liposomes obtained from the lipid mixing assay (Figure 4) is also presented (●).

and protoplasts was demonstrated with liposomes containing calcein at self-quenching concentrations (Allen & Cleland, 1980; Connor et al., 1984). Figure 5 shows the time dependence of the content mixing during the interaction of calcein-containing liposomes with protoplasts at pH's 3.9 and 5.6. Before addition of the protoplasts, the medium containing liposomes encapsulating calcein is fluorescent—presumably due to the fluorescence of calcein in liposomes and/or to liposome leakage. The addition of protoplasts first produces an abrupt reduction of the calcein fluorescence through light-scattering and filter effects (corresponding to time zero in Figure 5). After this reduction, the calcein fluorescence begins to increase if liposomes release their contents. This time course of the increase in calcein fluorescence in the cases when liposomes encapsulating calcein were reacted with rye protoplasts at two different pH values is presented in Figure 5. The fast enhancement of calcein fluorescence when the reaction was performed at pH 3.94 (Figure 5, curve 1) is seen by comparison with curve 2 (for the experiment in curve 2, the pH of the reaction medium was 5.64). Using the calcein assay, we studied the pH dependence of the interaction of liposomes with protoplasts. Thus, experiments similar to those presented in Figure 5 were performed at different pH's. From the kinetic curves obtained, the half-times for content mixing ($t_{1/2}^c$) were determined. These half-times were strongly pH dependent (Figure 6) and are similar to the half-times measured by the lipid mixing assay.

Fluorescence microscopic observations demonstrated that the protoplasts exhibited a strong, diffuse fluorescence in the cytoplasm after fusion. Further evidence that the increase in calcein fluorescence in the liposome–protoplast fusion experiments (i.e., Figure 5) is the result of dequenching of the calcein fluorescence inside the protoplast was obtained by the following experiments: Liposomes encapsulating calcein were incubated with protoplasts at different pH values (10 min, 30 °C, see Materials and Methods). After the suspension was washed by two centrifugations, the calcein fluorescence associated with the protoplast pellets (before and after adding 2% Triton X-100) was measured. From these values the percent of fused liposomes from the total amount of liposomes associated with the protoplast pellets was calculated by use of eq 5 (see Materials and Methods). Thus, the pH profile of the percent fused liposomes was obtained [Figure 7, (○)]. In Figure 7 we present also the values of percent fusion of liposomes and protoplasts measured from the lipid mixing assay [(●), data from Figure 5]. Both assays give similar percent fusion values, which indicates that they reflect the same interaction process (i.e., the liposome–protoplast fusion). Another important result from the experiments presented in Figure 7 (●) is that calcein is not leaking from the protoplasts after fusion (calcein fluorescence is internalized in the protoplast). This result was also supported by fluorescence microscope observations.

In another set of experiments, protoplasts were incubated with high concentrations of calcein under the same conditions used in the liposome–protoplast fusion experiments. After being washed by two centrifugations, the protoplast pellets were analyzed microscopically and a very pale cytoplasmic fluorescence was observed. Further evidence that liposomes deliver their contents to the protoplast interior was obtained by the following experiments: high molecular weight fluorescein-labeled dextran molecules (156 kDa; FITC-D150) were also incorporated in the liposomes. After reaction of these liposomes at acidic pH with rye protoplasts, the protoplast pellet was washed and concentrated by two centrifugations. A strong diffuse and punctate fluorescence of FITC-D150 in

the cytoplasm was observed under the microscope. When protoplasts were incubated with free FITC-D150 at concentrations similar to those of FITC-D150 inside the liposomes, no fluorescence in the cytoplasm could be observed after washing.

Discussion

We have presented evidence that below pH 5 liposomes composed of a lipid mixture isolated from soybeans fuse with rye protoplasts. Mixing of membrane lipids was monitored by the resonance energy transfer method. For these experiments we used N-NBD-PE as the energy donor and N-Rh-PE as the energy acceptor since it was shown that these lipids are not exchanged between lipid vesicles either through the aqueous phase or in vesicle aggregates (Nichols & Pagano, 1981; Struck et al., 1981; Arvinte & Hildenbrand, 1984); also, no exchange was found with these labeled lipids in the interaction between liposomes and cells (Struck & Pagano, 1980; Nichols & Pagano, 1981; Wojcieszyn et al., 1983). In the situations when labeled or unlabeled lipids with long acyl chains are exchanged between liposomes and cells, this process is slow, the half-times being on the order of hours (Bloj & Zilversmit, 1976, 1977; Wojcieszyn et al., 1983). The relatively rapid mixing we observed ($t_{1/2} \sim 1$ min at 30 °C) is difficult to reconcile with exchange times reported in the literature for mechanisms other than fusion. A pH-facilitated transfer of these long-chain fluorescent lipids is also very unlikely because no N-NBD-PE or N-Rh-PE probe dilution was detected when labeled liposomes were mixed with unlabeled liposomes in the range between pH 3 and 8 (Driessen et al., 1985). For the temperature dependence of the half-times (Figure 2), an apparent activation energy of 21 ± 5 kcal/mol was obtained. This value is consistent with a fusion mechanism because it is in the same order or magnitude of kinetic data reported for the fusion of ganglioside-containing liposomes with Sendai virus (E_a = 26 kcal/mol) (Tsao & Huang, 1985), of Sendai virus with erythrocyte membranes (E_a = 22 ± 6 kcal/mol) (Lyles & Landsberg, 1979), and of liposomes bearing covalently bound lysozyme with erythrocyte ghosts (E_a = 28 ± 3 kcal/mol) (Arvinte et al., 1986). Therefore, the most probable origin of the lipid mixing is the fusion of the liposomes and the protoplast membrane. Mixing of lipids is a necessary, but not sufficient, criterion for proving that fusion of two membranes has occurred. Because there are cases where lipid mixing is not accompanied by content mixing (e.g., Ellens et al., 1985), it was necessary to examine whether, under our experimental conditions, there was mixing of the liposome contents with the protoplast cytoplasm.

A general method to study the transfer of liposome contents into a membrane-bound biological system (i.e., cell, nuclei, erythrocyte ghosts, etc.) is to encapsulate molecules in the liposomes and to prove that (i) after fusion the molecules are present in the biological systems and (ii) the molecules entered during fusion with liposomes and not through other processes. We used a method based on fluorescence enhancement of a concentrated solution of a fluorescent aqueous marker (self-quenching concentrations), initially confined in the liposomes. This method was originally developed for the dye carboxyfluorescein to study liposome–cell interactions (Weinstein et al., 1977). Our experiments performed with calcein showed that during the low-pH interaction of liposomes with rye protoplasts, a strong dequenching of the calcein took place (Figure 5). The kinetics of the calcein dequenching were fast (half-times about 1 min). The similarity of the half-times measured with the calcein dequenching (content mixing assay) and with the lipid resonance energy transfer assay is an indication that we monitored concomitant phenomena (liposome content release and mixing of liposomes with plasma membrane lipids). Fluorescence microscopy of rye protoplasts after incubation with calcein–liposomes showed a diffuse distribution of the dye fluorescence, almost certainly indicating release into the cytoplasm. Experiments employing protoplast pellets (Figure 7) showed that protoplasts retained a strong calcein fluorescence after interaction with liposomes and that the protoplasts were not leaky (conclusion supported also by fluorescence microscopy observations). Experiments with free calcein and free dextran (FITC-D150) showed that at high concentrations the amount of dyes that enter the protoplasts is very small. This suggested that it is very improbable that in our experiments the transfer of liposome contents to the protoplast interior took place by other mechanisms: i.e., leakage of liposomes at the cell surface and entry into the cell before diffusion out of the unstirred layer occurred (Allen et al., 1981; Kosolowski et al., 1978). This transfer of liposome contents to the protoplasts resulting from liposome leakage at the cell surface (the "unstirred layer") model (Allen et al., 1981; Koslowski et al., 1978) was also shown not to be the explanation of the uptake of liposome contents by murine tumor cells (Blumenthal et al., 1982). Thus, the calcein release experiments are strong evidence for the transfer of liposome contents into the protoplast cytoplasm.

In summary, this study shows that at low pH, liposomes fuse with the plasma membrane of isolated plant protoplasts. Consequently, the lipid composition of the plasma membrane of protoplasts can be modified. Current studies are directed to the cryobehavior of the plasma membrane of protoplasts after fusion with liposomes of differing composition.

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References

Allen, T. M., & Cleland, L. G. (1980) *Biochim. Biophys. Acta 587*, 418–426.

Amselem, S., Barenholz, Y., Loyter, A., Nir, S., & Lichtenberg, D. (1986) *Biochim. Biophys. Acta 860*, 301–313.

Arvinte, T., & Hildenbrand, K. (1984) *Biochim. Biophys. Acta 775*, 86–94.

Arvinte, T., Hildenbrand, K., Wahl, P., & Nicolau, C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 963–966.

Arvinte, T., Wahl, P., & Nicolau, C. (1987a) *Biochemistry 26*, 765–772.

Arvinte, T., Wahl, P., & Nicolau, C. (1987b) *Biochim. Biophys. Acta 899*, 143–150.

Bloj, B., & Zilversmit, D. B. (1976) *Biochemistry 15*, 1277–1283.

Bloj, B., & Zilversmit, D. B. (1977) *Biochemistry 16*, 3943–3948.

Blumenthal, R., Ralston, E., Dragsten, P., Leserman, L. D., & Weinstein, J. N. (1982) *Membr. Biochem. 4*(4), 283–303.

Connor, J., Yatvin, M. B., & Huang, L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1715–1718.

Dowgert, M. F., & Steponkus, P. L. (1984) *Plant Physiol. 75*, 1139–1151.

Driessen, A. J. M., Hoekstra, D., Scherphof, G., Kalicharan, R. D., & Wilschut, J. (1985) *J. Biol. Chem. 260*, 10880–10887.

Ellens, H., Bentz, G., & Szoka, F. C. (1985) *Biochemistry 24*, 3099–3106.

Fernandez, S. M., Lurquin, P. F., & Kado, C. I. (1978) *FEBS Lett. 87*, 277–282.

Fraley, R. T., Dellaporta, S. U., & Papahadjopoulos, D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1859–1963.

Freeman, J. P., Draper, J., Devey, M. R., Cocking, E. C., Gartland, K. M. A., Harding, K., & Pental, D. (1984) *Plant Cell Physiol. 25*, 1353–1365.

Fukunaga, Y., Nagata, T., & Takebe, I. (1981) *Virology 113*, 752–760.

Fukunaga, Y., Nagata, T., Takebe, I., Kakehi, T., & Matsui, C. (1983) *Exp. Cell Res. 114*, 181–189.

Fung, B. K.-K., & Streyer, L. (1978) *Biochemistry 17*, 5241–5248.

Garcia, A. F., & Drew, G. (1984) *Z. Naturforsch., C: Biosci. 39C*, 1112–1119.

Gordon-Kamm, W. J., & Steponkus, P. L. (1984a) *Protoplasma 123*, 83–94.

Gordon-Kamm, W. J., & Steponkus, P. L. (1984b) *Protoplasma 123*, 161–173.

Gordon-Kamm, W. J., & Steponkus, P. L. (1984c) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6373–6377.

Hoekstra, D. (1982) *Biochim. Biophys. Acta 692*, 171–175.

Koslowski, M., Rosen, F., Milholland, R., & Papahadjopoulos, D. (1978) *Cancer Res. 38*, 2848–2853.

Lyles, S., & Landsberger, F. R. (1979) *Biochemistry 18*, 5088–5095.

Lynch, D. V., & Steponkus, P. L. (1987) *Plant Physiol. 83*, 761–767.

Nagata, T. (1984) in *Cell Fusion: Gene Transfer and Transformation* (Beers, R. F., Jr., & Bassett, E. G., Eds.) pp 217–226, Raven, New York.

Nagata, T., Okada, K., Takebe, I., & Matsui, C. (1981) *MGG, Mol. Gen. Genet. 184*, 161–165.

Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783–2789.

Pihakaski, K., & Steponkus, P. L. (1987) *Physiol. Plant. 69*, 666–674.

Rollo, F., Galli, M. G., & Parisi, B. (1981) *Plant Sci. Lett. 20*, 347–354.

Schneider, H., Lemasters, J. J., Höchli, M., & Hackenbrock, C. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3273–3277.

Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1985) *Biochemistry 24*, 3107–3113.

Steponkus, P. L. (1984) *Annu. Rev. Plant Physiol. 35*, 543–584.

Struck, D. K., & Pagano, R. E. (1980) *J. Biol. Chem. 225*, 5404–5410.

Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.

Tsao, Y. S., & Huang, L. (1985) *Biochemistry 24*, 1092–1098.

van Meer, G., & Simons, K. (1983) *J. Cell Biol. 97*, 1365–1374.

Wang, C.-Y., Hughes, K. W., & Huang, L. (1986) *Plant Physiol. 82*, 179–184.

Weinstein, J. N., Yoshikami, S., Henhart, P., Blumenthal, R., & Hagins, W. A. (1977) *Science (Washington, D.C.) 195*, 489–492.

White, J., & Helenius, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3273–3277.

Wojcieszyn, J. W., Schlegel, R. A., Lumley-Sapansky, K., & Jacobson, K. A. (1983) *J. Cell Biol. 96*, 151–156.