Parameters Influencing the Liposome-Mediated Insertion of Fluorescein Diacetate into Plant Protoplasts

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ABSTRACT

Maximum uptake of liposome-encapsulated fluorescein diacetate by Daucus carota protoplasts was observed when 6 x 10^6 protoplasts per milliliter were incubated with 2.4 x 10^6 liposomes per milliliter for 1 hour. In the case of Nicotiana glutinosa protoplasts, optimum ratio of protoplasts to liposomes was 1:10, where 2.3 x 10^6 protoplasts per milliliter were provided. Neutral and positive liposomes were found to be efficient vehicles to transfer their contents into plant protoplasts. When protoplasts treated with liposomes were cultured in a synthetic medium for 1 week, 20% resumed cell divisions.

Liposomes (lipid vesicles) have been used to encapsulate various materials ranging from small molecules, such as ions, chelating agents, sugars, and drugs, to macromolecules, including proteins, RNA, and even chromosomes (6, 9). Most of the published work on liposome-mediated transfer of materials into living cells or tissues deals with animal systems.

Nevertheless, there has been presented some evidence showing incorporation of liposome-sequestered materials into plant protoplasts. Cassells (2) demonstrated that tomato leaf protoplasts took up large charged vesicles, 2 to 6 μm in diameter. Furthermore, Matthew et al. (6) reported their systematic work on the uptake of liposome-encapsulated Escherichia coli RNA by carrot protoplasts.

It has been hoped that liposomes will serve as potential vehicles for transfer of undegraded forms of genetic materials into plant protoplasts. To be applicable for the purpose of genetic manipulation of plant cells, appropriate systems whereby an efficient uptake of liposome-encapsulated materials by plant protoplasts occurs need to be established. From this point of view, fundamental problems associated with the interaction between lipid vesicles and plant protoplasts must be solved. Such a basic approach may provide a valuable insight into developing a new technology for plant improvement. Thus, I was prompted to establish conditions capable of introducing a measurable amount of liposome-contents into plant protoplasts.

MATERIALS AND METHODS

Protoplasts. Nicotiana glutinosa protoplasts and Daucus carota protoplasts were prepared from suspension cultured cells according to a method reported elsewhere (10).

Liposomes. Fifteen mg hydrogenated phosphatidylcholine (egg lecithin) and 1.5 mg stearylamine dissolved in 2 ml chloroform, which was contained in a 50-ml, round-bottom flask. Chloroform was evaporated under N₂ gas with a gentle rotation of the flask. In one experiment, dicetyl phosphate was added to the egg lecithin. Egg lecithin, dicetyl phosphate, and stearylamine were purchased from P. S. Biochemical. After complete drying of a film made on an inside wall of a flask, 2 ml 50 mM phosphate buffer (pH 7.0) containing 0.5 mM mannitol and 0.01% FDA was added. Then the flask was vigorously shaken by hand for 5 min. Large vesicles containing FDA (liposome-FDA) were pelleted with centrifugation at 500g for 5 min. Small vesicles and unsequestered FDA were retained in the supernatant and discarded. Vesicles were washed with phosphate buffer containing 0.5 mM mannitol at least three times.

Incubation of Liposomes and Protoplasts. Fifty μl of protoplast suspension was mixed with an equal volume of liposome-FDA in a 0.8- x 7.5-mm test tube. Unless otherwise mentioned, incubation mixture contained 10⁵ to 10⁶ protoplasts/ml and 10⁴ to 10⁵ liposome-FDA/ml. Incubation was carried out using a drum rotating at 1 rpm. After a given period of incubation at 25 C, protoplast and liposome-FDA mixtures were centrifuged at 150g for 1.5 min, and pelleted protoplasts were resuspended in 0.5 mM mannitol solution. Protoplasts thus were washed at least three times.

Observation of Fluorescent Staining of Protoplasts Caused by Liposome-mediated Transfer of FDA. Fluorescence of protoplasts was observed using Olympus fluorescent microscope equipped with excit filter BG 12 and barrier filter 47 (Fig. 1). FDA stains only living cells due to the catalytic action of endogenous esterase (5). Protoplasts containing liposome-contents were expressed as a per cent of total protoplasts, where at least 150 protoplasts were observed in each test.

RESULTS AND DISCUSSION

Time Course of Uptake of Liposome Contents by Protoplasts. Liposomes were incubated with either D. carota or N. glutinosa protoplasts for 3 h. Efficiency of liposome-mediated transfer of FDA into protoplasts reached a peak after 1 h incubation period in both protoplast types (Fig. 2). Maximum observed FDA release into N. glutinosa protoplasts was 35%, whereas it was 47% in D. carota protoplasts. Using large vesicles prepared by a similar method as in this paper, Cassells (2) observed a release of fluorescent contents in central vacuoles of tomato leaf protoplasts after 15 h incubation. Cassells used 1 x 10⁵ protoplasts/ml to incubate with the same concentration of liposomes. The efficiency of the uptake of liposome contents by plant protoplasts may depend on the ratio between liposomes and protoplasts; thus, optimum ratio of liposomes to protoplasts was determined in the following experiment.

Ratio of Protoplasts to Liposomes. In this experiment, ratio of protoplasts to lipid vesicles was varied, where either N. glutinosa protoplasts at 2.3 x 10⁵/ml or D. carota protoplasts at 6 x 10⁵/ml were kept constant. When more than 10 times lipid vesicles were added to protoplasts, over 70% D. carota protoplasts appeared to have absorbed liposome contents (Fig. 3). In the case of N. glutinosa, maximum uptake of liposome-encapsulated FDA was

1 Abbreviation: FDA, fluorescein diacetate.
lecinthin. Negatively charged vesicles were obtained by mixing 21 μmol lecinthin and 6 μmol dicetyl phosphate. The result summarized in Table I demonstrates that tobacco protoplasts took up neutral vesicles much more readily than charged vesicles in 1-h incubation. On the contrary, there was no significant difference between neutral and positive vesicles with respect to an efficiency of liposome-contents uptake by carrot protoplasts. In both cases, negatively charged vesicles resulted in the lowest uptake efficiency by protoplasts. Inasmuch as surface charge of plant protoplasts was reported to be negative (1, 8), incidence of the fusion of negatively charged vesicles with plant protoplasts is unlikely to exceed that of positive vesicles. Furthermore, the differences in the efficiency of the transfer of liposome contents between carrot and tobacco protoplasts could have resulted from the quantitative differences of the surface membrane of the two species.

**Competition Experiment using Unlabeled Liposomes.** D. carota protoplasts were mixed with liposome-FDA, where liposomes without FDA (unlabeled liposomes), were added up to 20 times more than liposome-FDA. Figure 4 indicates that unlabeled liposomes did not interfere with the liposome-FDA uptake by protoplasts. Therefore, liposomes do not seem to have specific binding sites on a membrane surface of protoplasts or at least, possible specific sites were not saturated.

**Size Distribution of Fluorescing Protoplasts.** Inasmuch as the size of protoplasts is not uniform among the protoplast population employed in this investigation, it might be reasonable to imagine
LIPOSOME-FDA TRANSFER INTO PROTOPLASTS

FIG. 3. Ratio of protoplasts to liposomes. Densities of *N. glutinosa* (N.G.) and *D. carota* (D.C.) protoplasts were kept constant at 2.3 x 10^5/ml and 6 x 10^6/ml, respectively, where concentrations of liposomes were varied. Experimental conditions and observations of liposome-mediated transfer of FDA into protoplasts were same as described in the legend of Figure 2.

that only a particular protoplast population may take up liposome contents. Thus, size distribution of fluorescing protoplasts, namely those which took up liposomes, was examined in both protoplasts types. The average diameter of *D. carota* protoplasts was 26.8 μm, whereas that of fluorescing *D. carota* protoplasts was 29.6 μm (Fig. 5). Similar results were also obtained with *N. glutinosa* protoplasts (data not presented). Therefore, size of protoplasts is not a responsible factor for the efficiency of liposome-mediated transfer of fluorescent material into protoplasts.

**Influence of Temperature.** To examine if energy-requiring processes are associated with the uptake of liposome contents by protoplasts, a mixture of protoplasts and liposomes was subjected to various temperature treatment. As shown in Table II, temperature ranging from 0 to 35 C did not influence the efficiency of liposome-mediated transfer of contents into protoplasts. Some-
what higher temperature, 45°C, stimulated the uptake of liposome-encapsulated contents by protoplasts. As suggested by Matthews et al. (6), high temperature may change the volume of the membrane, which consequently causes release of liposome contents into the protoplasts.

Another experiment (data not shown) indicates that the respiratory inhibitor, potassium cyanide at 1 mM, did not inhibit uptake of liposome contents by protoplasts. These evidences may suggest that the mechanism for uptake of liposomes by protoplasts did not solely involve endocytosis.

**Influence of PEG on Uptake of Liposome Contents by Protoplasts.** PEG is a known reagent which is capable of inducing protoplast fusion (3, 4). Thus, influence of various concentrations of PEG (mol wt, 6,000) to the liposome (consisting of egg lecithin alone)-FDA uptake by D. carota protoplasts was investigated. Low concentration of PEG at 1% seems to stimulate a release of liposome-contents into carrot protoplasts, whereas PEG concentrations in the range of 5 to 20% did not (Fig. 6). PEG at its high concentration did not stimulate the transfer of liposome contents into D. carota protoplasts. The action of PEG in membrane fusion seems to be somewhat ambiguous. Thus, understanding the precise mechanism associated with liposome-plant membrane interaction will be a future subject of research.

**Viability of Protoplasts Treated with Liposomes.** Upon the culture of *N. glutinosa* protoplasts without liposome treatment (3, 7), about 25% of protoplasts resumed cell divisions after 1 week culture. Under the same culture condition, liposome-treated protoplasts resulted in 20% cell division. Thus, viability of protoplasts was not significantly lowered by liposome treatment.

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**LITERATURE CITED**