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**Entrapment of plasmid DNA by liposomes and their interactions with plant protoplasts**

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**ABSTRACT**

Lecithin and lecithin/cholesterol liposomes formed in aqueous solutions of DNA entrap covalently closed circular, open circular and linear DNA molecules of size up to at least 13 kilobases. The sequestered DNA molecules are efficiently protected against exogenous deoxyribonuclease action although nicking and linearization of circular DNA can be observed. The size of these liposomes ranges from approximately 0.5 to 7.5  $\mu$  with an average of 2.5 - 4  $\mu$ . DNA filled liposomes strongly interact with plant protoplasts under conditions inducing protoplast fusion. Results suggest that sequestered plasmid DNA can be transferred to protoplast nuclei.

**INTRODUCTION**

Large liposomes have been successfully used in the transfer of biologically active m-RNA (1) and metaphase chromosomes (2) to cultured mammalian cells. Furthermore, it was shown recently that high molecular weight DNA could also be entrapped by similarly prepared liposomes (3).

The introduction of new genetic information into plant protoplasts is a major issue from the viewpoint of plant somatic cell molecular genetics and hereditary modification of plants (4-8). Thus, our interest is to insert exogenous DNA into plant protoplasts in order to assess its fate and biological activity after uptake. Obviously, a prerequisite for the biological expression of foreign DNA is the existence of a system allowing uptake of undegraded donor DNA. Unfortunately, one of the main problems associated with this type of experiments is the release of deoxyribonuclease activity by plant protoplasts into the incubation medium (9-11). Degradation of donor DNA in the medium can be partially prevented by a variety of methods involving complex formation between DNA and polycations as well as incubation of protoplasts and DNA at high pH values (9, 12-15). It appeared to us that entrapment of DNA by liposomes would not only protect it against deoxyribonuclease attack but might also enhance its uptake

i.e. as a result of liposome-protoplast adhesion or fusion. Indeed, comparatively large bodies bounded by biological membranes such as bacteria and chloroplasts can be engulfed by plant protoplasts after surface activation (16, 17). On the other hand, plasmid DNA molecules constitute a good model system in DNA uptake experiments owing to their resistance to exonuclease activity also released by plant protoplasts (15, 18) and above all thanks to their properties as cloning vehicles. Indeed, one may anticipate that future plant vectors will be circular DNA molecules (7).

In this paper we present evidence for plasmid and chromosomal DNA entrapment by lecithin and lecithin/cholesterol liposomes using radio-labeled DNA as well as fluorescent dyes-DNA complexes. We discuss the effects of exogenously added deoxyribonuclease and plant protoplasts exsudes on the integrity of sequestered DNA molecules. The location and physical state of donor DNA after liposome-protoplast interaction is also investigated. Part of these results were briefly reported elsewhere (P.F. Lurquin, Arch. Int. Physiol. Biochim., in press).

### MATERIALS AND METHODS

#### Preparation of bacterial DNA

Unlabeled *Escherichia coli* CR 34 chromosomal DNA was prepared as described in (19). It was needle-sheared to a molecular weight of approx. 7 M daltons as determined by viscometry (20). [<sup>3</sup>H]-thymidine labeled pBR322 (2.6 M daltons, approx. 4 kilobases) and pCR1 (8.6 M daltons, approx. 13 kilobases) plasmids harbored in *E. coli* RR1 and C600 respectively were prepared as described before (9, 13). Their specific radioactivities were 254,800 cpm/μg (pBR322) and 42,900 cpm/μg (pCR1).

#### Preparation of protoplasts, nuclei and nucleic acids extracts.

Cowpea (*Vigna sinensis* cv. Blackeye 5) seeds were germinated and grown for 7 days in Shell Kerex mould (27°C, 75% humidity, 10,000 lux, 16 hour photoperiod). Fully expanded primary leaves were used for enzymatic protoplast isolation as described in (21) except that a hard nylon brush instead of carborundum was used to remove the lower epidermis. All operations dealing with protoplast isolation and handling were carried out in a laminar flow cabinet. Protoplasts were suspended in Gresshoff-Doy medium (pH 5.6) containing 0.45 M mannitol to a concentration of  $6-7 \times 10^6$  per ml. Nuclei were isolated and purified as in (9) except that additional washings were done with buffered sucrose solutions containing 1%, 0.5% and 0% Triton

X-100. Total nucleic acids extracts from whole protoplasts, the "cytoplasmic" and nuclear fractions were obtained by lysing protoplasts and nuclei with 2% sodium dodecylsulfate in 0.1M Tris-HCl, 0.2M NaCl (pH 9.0) followed by a 1 hour incubation at 37° in the presence of 100 µg/ml proteinase K (Merck). Deproteinization was performed twice with an equal volume of phenol (equilibrated with the above pH 9.0 buffer)/chloroform/isoamyl alcohol 50:50:1. Nucleic acids were then precipitated with two volumes of cold ethanol and redissolved in 10 mM Tris-HCl buffer pH 7.5.

#### Preparation of liposomes.

Lecithin (L- $\alpha$ -phosphatidyl choline from egg yolk, type V-E, Sigma) and lecithin/cholesterol (Sigma) liposomes were prepared as in (3) and in (2) by evaporating in a vacuum and at room temperature a chloroform solution of lecithin or lecithin and cholesterol (ratio 7:2 w/w) in a round bottom test tube. DNA dissolved in 1 ml 0.02M Tris-HCl, 1 mM MgCl<sub>2</sub> pH 7.5 (liposome buffer) was shaken for 90 min. at 27° with the lipid film at a DNA : lipid ratio of approx. 500. Liposomes were sedimented at 15,000 rpm for 10 min. at room temperature in a SS34 Sorvall rotor and washed once with liposome buffer.

Fluorescence microscopic examinations were performed on liposomes containing preformed ethidium bromide (EB) - *E. coli* chromosomal DNA complexes (10 µg EB added to 20 µg in 1 ml liposome buffer) and 4',6-diamidino-2-phenylindole (DAPI) - *E. coli* DNA complexes (20 µg DAPI and 20 µg DNA in 1 ml liposome buffer). Photographs were taken with a Zeiss microscope equipped with a UV fluorescence attachment. Size determination was performed on color transparencies of fluorescent liposomes with a MOP-AM03 digitizer (Kontron, German Federal Republic).

#### Incubation of protoplasts.

Protoplasts resuspended as above were supplied either with free [<sup>3</sup>H]-pBR322, [<sup>3</sup>H]-pBR322 complexed with poly-L-ornithine (Miles) at a DNA/polycation ratio of 3:2 (w/w) or [<sup>3</sup>H]-pBR322 sequestered in lecithin liposomes. In the latter case, 2 volumes of 0.037M polyethylene glycol 6000 (PEG) in 4.7 mM CaCl<sub>2</sub>, 0.1 M glucose pH 5.6 were sometimes added to protoplasts pretreated with [<sup>3</sup>H]-pBR322-containing liposomes. This treatment causes adhesion and fusion of protoplasts (22). Incubation with DNA and liposomes was for 45 min. at room temperature. In other experiments, [<sup>3</sup>H]-pBR322 was incubated with protoplasts in high pH medium in the presence of 50 mM CaCl<sub>2</sub>, 0.45 M Mannitol and 50 mM glycine-NaOH buffer pH 10.0 (23). The concentration of [<sup>3</sup>H]-pBR322

was between 1 and 6.7  $\mu\text{g/ml}$ . After the incubation, protoplasts were washed 4 times with 5 ml incubation medium and processed for nuclei or nucleic acids extraction. The incubation medium containing unbound DNA or liposomes was kept in order to estimate by difference the amount of DNA and liposomes both weakly and strongly bound to protoplasts as well as its degree of polymerization.

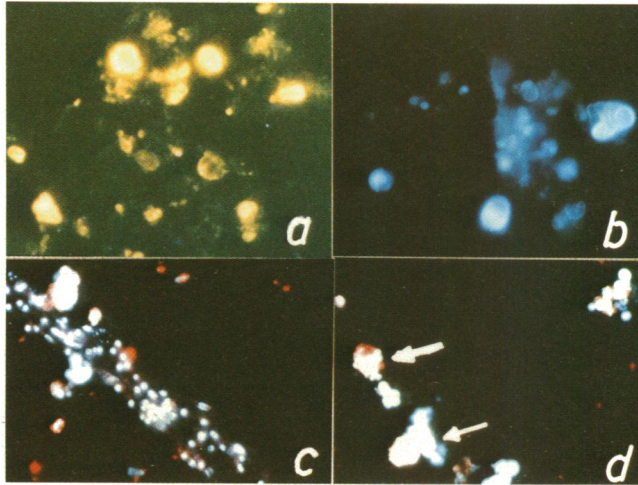
### DNA analysis.

The degree of polymerization of plasmid DNA recovered from liposomes disrupted with 2% Triton X-100 (British Drug House) in liposome buffer was estimated by agarose gel electrophoresis (0.8% agarose from Industrie Biologique Française in 40 mM Tris base, 20 mM sodium acetate and 2 mM disodium EDTA) in 14 cm long cylindrical gels at 100 Volts and 4 mA/gel. Electrophoresis was stopped when the bromophenol blue marker had reached the bottom of the tube. The gels were then sliced into 2 mm thick fractions. Similarly, nucleic acids extracts from plasmid treated protoplasts were analyzed by molecular sieving and gel electrophoresis. The amount of donor- $[^3\text{H}]$  DNA bound to protoplasts - regardless of its degree of polymerization - was measured by precipitating protoplast lysates (9) with 2 volumes of cold 10% trichloroacetic acid (TCA) and collecting the precipitates on GF/C glass fiber filters (Whatman). Radioactivity determinations were done by liquid scintillation (9, 19) with a Packard TriCarb B2450 Liquid Scintillation Spectrometer.

## RESULTS AND DISCUSSION

### a. Characterization of DNA-containing liposomes.

DNA is able to form stable complexes with aromatic compounds such as EB and DAPI. Quite interestingly, complex formation is in both cases accompanied by a very large enhancement of the fluorescence of these drugs (24,25). When lecithin liposomes are formed in the presence of DNA-fluorochrome complexes, washed with liposome buffer and observed under the fluorescence microscope, it can be seen that the vesicles brightly fluoresce orange (with EB-DNA) or blue (with DAPI-DNA) (Figure 1). No background fluorescence attributable to unsequestered DNA is detectable. It is found that the size of these liposomes is quite variable. Measurements carried out on 171 fluorescent vesicles show that liposome diameters range from 0.5 to 7.5  $\mu$ , most particles being distributed between values of 2.5 - 4  $\mu$ . Since lecithin and lecithin/cholesterol liposomes are found to bind the same amount of DNase-resistant



**Fig. 1.** Fluorescence micrographs of DNA-filled liposomes and liposomes interacting with cowpea protoplasts. *a.* Liposomes loaded with a DNA-ethidium bromide complex. *b.* Liposomes loaded with a DNA-DAPI complex. *c.* and *d.* DNA-DAPI loaded liposomes (blue-white dots) attached to cowpea protoplasts after incubation with PEG. In most cases separated liposomes are seen (*c* and *d*, upper arrow), sometimes the fluorescent complex diffuses throughout the protoplast (*d*, lower arrow), *a* and *b* x2000; *c* and *d* x200.

plasmid DNA (not shown), only lecithin liposomes will be considered below. Moreover, the quantitative aspects of plasmid DNA sequestration are close to the values reported in (3).

The use of covalently closed circular DNA in these experiments allows a fine analysis of the physical integrity of sequestered DNA since single site nicking can easily be detected by agarose gel electrophoresis (27). Figure 2 shows that our original [ $^3\text{H}$ ]-pBR322 DNA preparation contains about 65% covalently closed circular (CCC) molecules, 33% open circular (OC) molecules and an estimated 2% linear (L) molecules. After liposome formation and washing in low ionic strength liposome buffer, CCC molecules represent 47% of total, OC molecules 45% and L molecules 8%. Thus, entrapment by liposomes induces some nicking and linearization of the DNA, possibly as a result of prolonged shaking necessary for liposome formation. After a high ionic strength wash to remove external DNA as in (3), the relative proportions of CCC, OC and L molecules are 40, 52 and 8% respectively. Finally, although DNase treatment (50  $\mu\text{g}/\text{ml}$  pancreatic DNase, 30 min. at 25°C) of plasmid-filled liposomes does not generate lower molecular weight products, it is

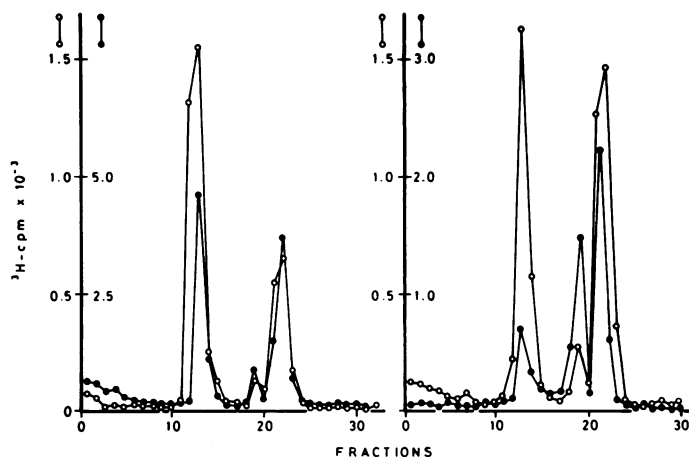
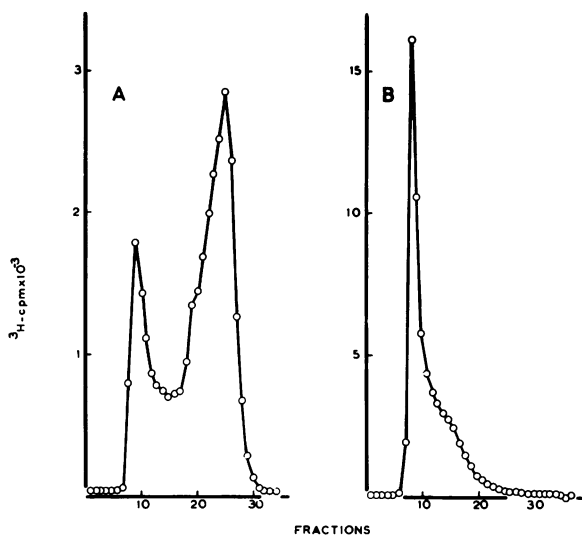


Fig. 2. Agarose gel electrophoresis of [ $^3\text{H}$ ]-pBR322 DNA recovered from liposomes lysed with 2% Triton X-100. Left O—O, untreated plasmid; ●—●, plasmid recovered from liposomes after low ionic strength washing. Right O—O, plasmid recovered from liposomes after washing at low and high ionic strength; ●—●, plasmid recovered from liposomes after washing at low and high ionic strength followed by incubation in the presence of pancreatic deoxyribonuclease as described in the text. Left, middle and right peaks represent covalently closed, linear and open circular DNA respectively.

clear that sequestered DNA remains somewhat exposed to nuclease attack since the proportion of CCC DNA is reduced to 20% (OC DNA = 51%, L DNA = 29%) after DNase treatment. Nevertheless plasmid DNA molecules are remarkably well protected by lipid membranes under the conditions used. Qualitatively similar results are obtained with [ $^3\text{H}$ ]-pCR1 (8.6 M dal).

#### b. Interactions between DNA loaded-liposomes and cowpea protoplasts.

Plant protoplasts excrete deoxyribonuclease activity into the medium (9, 11, 15, 28, 29). Cowpea mesophyll protoplasts, although releasing less DNase activity than tobacco mesophyll protoplasts, actively degrade exogenous DNA in the culture medium (15). A 45 minute incubation of cowpea protoplasts with [ $^3\text{H}$ ]-pBR322 sequestered in lecithin liposomes leaves unbound excess DNA in the medium. Centrifugation of this medium (after harvesting the protoplasts) at 27,000 x g for 10 min. yields a liposome pellet and a supernatant fraction. Figure 3A shows the analysis by molecular sieving on Sepharose 4B of DNA found in the supernatant. It can be seen that about 30% of the radioactivity is eluted at the void volume, the remaining 70%



**Fig. 3.** State of [ $^3\text{H}$ ]-pBR322 donor DNA remaining in the medium after incubation with cowpea protoplasts and plasmid sequestered in liposomes. Molecular sieving on Sepharose 4B. (19). A. DNA not sedimenting at 27,000xg for 10 minutes. B. DNA sedimenting together with liposomes at 27,000xg. The pellet was treated with 2% Triton X-100 before analysis.

being eluted at the position of highly depolymerized DNA. This result indicates that cowpea protoplasts possess the ability to disrupt lecithin membranes and that DNA released from these vesicles becomes susceptible to nuclease attack. However, most (ca. 70%) of the total radioactivity found in the medium sediments together with the liposomes and has retained a high degree of polymerization as indicated in fig. 3B. Taken together, the radioactivity values found to elute at the position of the void volume in fig. 3A and B represent ca. 80% of the total radioactivity remaining in the incubation medium. Under similar conditions, if supplied as free molecules, excluded DNA only represents ca. 40% of the total after incubation with protoplasts (not shown).

Cassells (30) has recently shown that lipid vesicles are able to fuse with tomato protoplasts in such a way that their contents can be released intracellularly. The following experiments are designed to determine whether sequestered plasmid DNA can also be transferred to the intracellular compartments of cowpea protoplasts.

Preliminary experiments (15) indicated that large amounts of [ $^3\text{H}$ ]-pBR322 DNA entrapped in lecithin liposomes were bound to cowpea protoplasts in the

presence of polyethylene glycol (PEG), an agent which causes protoplast fusion. Table I shows that PEG causes extensive removal of DNA containing liposomes from the incubation medium and subsequent attachment - in an as yet undetermined way - to protoplasts. Similarly, these conditions allow the highest recovery of TCA-precipitable radioactivity from extensively washed protoplasts (Table I) compared to other methods known to enhance DNA uptake by protoplasts. Moreover, figures 1C and D show fluorescence micrographs of liposomes loaded with a DNA-DAPI complex (blue dots) tightly bound to protoplasts recognizable by the weak red fluorescence of chlorophyll.

However, these observations do not allow a distinction between actual uptake of liposomes into protoplasts and their tight but external binding to the protoplast membrane. In order to distinguish between these possibilities, treated protoplasts were lysed with Triton X-100 and fractionated into a "cytoplasmic" and a nuclear fraction as indicated in Materials and Methods. In order to avoid a possible redistribution between pBR322 DNA

Table I. Binding of [ $^3$ H]-pBR322 to cowpea protoplasts under various experimental conditions.

	Control (no addition)	PL0-Zn <sup>++</sup> (a)	High pH-Ca <sup>++</sup> (b)	Liposomes	Liposomes <sup>(c)</sup> + PEG
Input cpm	350,000	350,000	350,000	399,000	399,000
Radioactivity remaining in the medium after the incubation (cpm)	221,060	47,030	41,560	227,780	42,300
Radioactivity as- sociated with the protoplast pellet (cpm)	8,850	47,840	16,470	18,860	105,610

(a) Plasmid DNA complexed with 2  $\mu$ g/ml poly-L-ornithine in the presence of 5 mM ZnSO<sub>4</sub> prior to incubation with protoplasts (9).

(b) Incubation at high pH in presence of 50 mM CaCl<sub>2</sub> at pH 10.0 as indicated in Materials and Methods and in (13), (15) and (23).

(c) Liposomes and protoplasts mixed with polyethylene glycol as described in Materials and Methods and in (22).



released from liposomes by the detergent and i.e. the nuclear membrane, the lysis was performed after addition of a very large (300  $\mu\text{g/ml}$ ) excess of unlabeled sheared *E. coli* chromosomal DNA (9).

These conditions virtually eliminate non-specific binding of DNA to nuclei as indicated by the following reconstruction experiment. When protoplasts are mixed in 1 ml with 300  $\mu\text{g}$  *E. coli* sheared chromosomal DNA and [ $^3\text{H}$ ]-pBR322 containing liposomes (representing an input of 390,000 cpm), immediately lysed with Triton X-100 and washed as in Materials and Methods, then, only 200 cpm are found associated with the nuclear fraction (0.05% of input). Typical experiments in which protoplasts are incubated for 45 min. with liposomes and PEG and then lysed in the presence of *E. coli* DNA yield values around 3% of input radioactivity associated with nuclei.

The following experiments were designed in order to determine the relative amounts of donor DNA present in the "cytoplasmic" and nuclear fractions as well as its degree of polymerization. Figure 4A represents the elution pattern on Sepharose 4B of a nucleic acids extract obtained from the "cytoplasmic" fraction of protoplasts treated with [ $^3\text{H}$ ]-pBR322 containing liposomes and PEG. It can be seen that most of the radioactivity is eluted later than the void volume (arrow) and thus re-

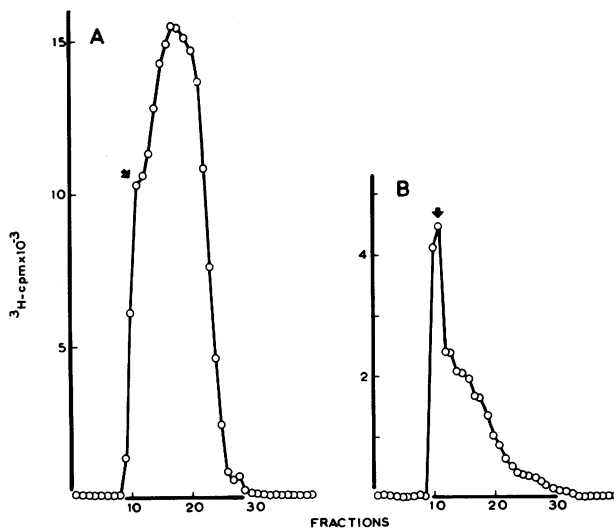


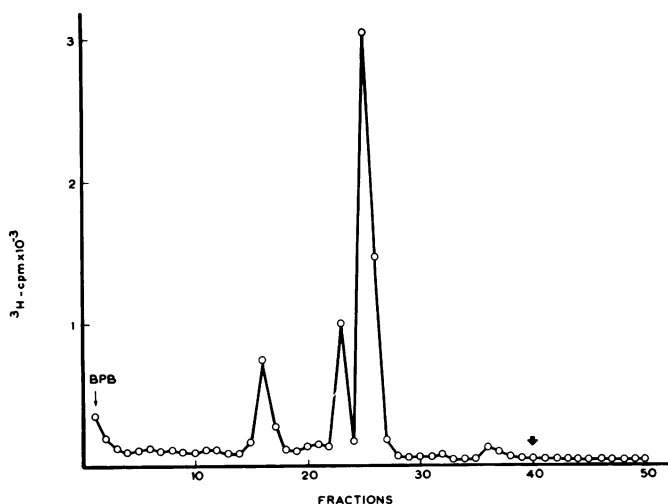
Fig. 4. State of [ $^3\text{H}$ ]-pBR322 donor DNA associated with protoplasts after incubation with plasmid-loaded liposomes. Molecular sieving on Sepharose 4B. A. Deproteinized "cytoplasmic" fraction. B. Deproteinized nuclear fraction. The arrows designate the void volume.

presents DNA molecules displaying various degrees of depolymerization. On the contrary, a significant proportion of the radioactivity associated with nuclei elutes at the void volume (fig. 4B), showing that plasmid molecules associated with nuclei undergo less depolymerization than those found in the cytoplasm. Moreover, the excluded peak shown in fig. 6B is found to contain supercoiled, linear and a majority of open circular [ $^3\text{H}$ ]-pBR322 as determined by electrophoretic analysis on 0.8% agarose (Fig. 5). The same figure shows that reutilization of donor DNA breakdown products for cellular DNA synthesis does not occur within the periods of incubation used. This is expected since it is known that freshly isolated protoplasts are unable to use thymine derivatives for DNA synthesis (31). Indeed, no radioactivity is found at the position of cowpea DNA in the gel (fig. 5, arrow).

## CONCLUSIONS

The experiments described here demonstrate that :

(1) large lecithin and lecithin/cholesterol liposomes can entrap plasmid DNA of molecular weight up to at least 8.6 M daltons, meaning that pieces of DNA of molecular weight around at least 6 M daltons could be cloned in a vehicle such as pBR322 (2.6 M daltons) and sequestered in these liposomes.



**Fig. 5.** Agarose gel electrophoresis of radioactive DNA associated with the nuclear fraction of protoplasts incubated with [ $^3\text{H}$ ]-pBR322 sequestered in liposomes. Peak designation as in figure 4 - BPB indicates the position of the bromophenol blue marker. The thick arrow indicates the position of cowpea nuclear DNA as revealed by staining with 1  $\mu\text{g/ml}$  ethidium bromide.

Cauliflower mosaic virus DNA, which has been considered to be a potentially useful plant host vector (32), could be recombined in vitro with a piece of DNA of molecular weight around at least 4.2 M daltons and be entrapped in the same way.

(2) sequestered DNA is efficiently protected against the action of purified pancreatic deoxyribonuclease and protoplasts exsudates possessing deoxyribonuclease activity.

(3) liposomes loaded with DNA tightly bind to cowpea protoplasts, especially in the presence of the fusion agent polyethylene glycol. Under those conditions, part of the donor DNA is found to be transferred to the nuclei in a relatively undegraded form.

Whether these molecules will be functionally active and whether some of them will be stably stored or integrated, are still unanswered questions, as is the question of the exact location of the plasmid molecules in the nuclei. The fact that degraded donor DNA is found in the cytoplasm of protoplasts incubated with liposomes suggests that the latter are able to disrupt lecithin membranes. That this phenomenon can occur intracellularly is shown by the finding that the ratio between donor DNA degradation products and high molecular weight DNA is higher in the protoplasts than in the incubation medium. These observations suggest actual uptake of donor DNA.

The biological activity of nucleic acids sequestered in liposomes could be tested in plant protoplasts systems where transfection with viral RNA (33) and viral DNA (34) have been demonstrated.

Finally, and from the point of view of plant genetic engineering, it will be of interest to check whether homologous or heterologous genes transferred by means of liposomes can be expressed in protoplasts from known plant biochemical mutants.

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