Liposome-Mediated Delivery of Deoxyribonucleic Acid to Cells: Enhanced Efficiency of Delivery Related to Lipid Composition and Incubation Conditions†

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ABSTRACT: Delivery of liposome-encapsulated simian virus 40 (SV40) DNA to African green monkey Related to been used as a probe to study liposome–cell interactions and to determine conditions which favor the intracellular delivery of liposome contents to cells. The efficiency of DNA delivery by various liposome preparations (monitored by infectivity assays) was found to be dependent both on the magnitude of vesicle binding to cells and on the resistance of liposomes to cell-induced leakage of contents. Acidic phospholipids were much more effective in both binding and delivery, and phosphatidylserine (PS) was the best in both aspects. The inclusion of 50 mol % cholesterol in liposomes reduces the cell-induced leakage of vesicle contents (2–5-fold) and substantially enhances the delivery of DNA to cells (2–10-fold). Following incubation of cells with negatively charged liposomes containing SV40 DNA, infectivity can be enhanced greatly by brief exposure of the cells to glycerol solutions. In contrast, only slight enhancement by glycerol was observed for SV40 DNA encapsulated in neutral or positively charged liposomes. The results of competition experiments between empty phosphatidylcholine liposomes and DNA-containing PS liposomes also suggest possible differences in the interaction of neutral and negatively charged liposome preparations with cells. Morphological studies indicate that the glycerol treatment stimulates membrane ruffling and vacuolization and suggest that the enhanced uptake of liposomes occurs by an endocytosis-like process. Results obtained with metabolic inhibitors are also consistent with the interpretation that the enhancement of liposome delivery in glycerol-treated cells occurs via an energy-dependent endocytotic pathway. Pretreatment of cells with chloroquine, a drug which alters lysosomal activity, further enhances infectivity in glycerol-treated cells (4-fold). This observation suggests the involvement of a lysosomal processing step at some point in the expression of liposome-encapsulated DNA and, more importantly, illustrates the possibility of altering cellular metabolism to engineer more efficient delivery by liposomes. Under optimal conditions determined in this study, the efficiency of liposome-mediated SV40 DNA delivery was increased more than 1000-fold over that obtained by simply incubating cells with liposomes. It is also demonstrated that these conditions enhance delivery of other molecules, besides DNA, which are encapsulated in liposomes.

In spite of the extensive work from several laboratories on the subject of liposome–cell interactions, it has been difficult to define unambiguously those conditions which favor increased intracellular uptake of liposome-encapsulated materials. In particular, the selection of an encapsulated marker for monitoring intracellular delivery has met with great difficulty. Radioactive compounds encapsulated in liposomes do not permit distinction between intracellular delivery of vesicle contents and the adsorption of intact liposomes to the cell surface (Pagano & Weinstein, 1978; Poste, 1980). A significant fraction of cell-associated liposomes remains adsorbed to cells during the first few hours of incubation (Szoka et al., 1980; Huang et al., 1977) and may eventually leak their contents into the external medium (Szoka et al., 1979). Consequently, fluorescent dyes and other molecules originally encapsulated in liposomes may be delivered intracellularly after their leakage from liposomes adsorbed to the cell surface and subsequent uptake by cell transport systems, transmembrane diffusion, or endocytosis.

Several studies have shown that proteins (Gad et al., 1979; Eytan & Eytan, 1980) or lipid derivatives (Martin & MacDonald, 1976; Struck & Pagano, 1980) incorporated into the liposome membrane can be transferred to cells in a functional form. However, their use as an assay for determining conditions which promote the uptake of encapsulated compounds by cells would be inappropriate since cellular incorporation of liposome membrane constituents may occur after the encapsulated contents have leaked out or by mechanisms which exclude concomitant cellular uptake of vesicle contents.

We have demonstrated that poliovirus RNA (Wilson et al., 1979; Papahadjopoulos et al., 1980a) and simian virus 40 (SV40)[1] DNA (Fraley et al., 1980; Papahadjopoulos et al., 1980b) can be encapsulated in large unilamellar phospholipid vesicles in a biologically active form. The viral nucleic acids are expressed when delivered intracellularly and sensitive plaque assays can be used to detect and quantify the generation of progeny virus. Thus, viral nucleic acids may be used as sensitive and discriminating probes for monitoring the intracellular delivery of liposomal contents and for determining the parameters which favor increased uptake.

Another important incentive for using nucleic acids as probes for the intracellular delivery of liposome contents is the potential of developing methods for increasing the functional

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[1] Abbreviations used: PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SA, stearylamine; Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; SV40, simian virus 40; pfu, plaque-forming units; SEM, scanning electron microscopy; AGMK, African green monkey kidney cells; poly(A), poly(deoxyadenylic acid); TBS, Tris-buffered saline; Cl_3CCOOH, trichloroacetic acid; MLV, multilamellar vesicles; REV, reverse-phase evaporation; CF, carboxyfluorescein.

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incorporation of RNA and DNA molecules into cells. Current techniques for introducing nucleic acids into cells (Graham, 1977; Pagano, 1969; Capecchi, 1980) are often very inefficient, limited to specific cell types, require large amounts of precious material, or, in the case of microinjection, allow manipulation of too few cells for many types of biochemical analysis. Liposome entrapment of nucleic acids is relatively simple and has resulted in the expression of RNA at higher efficiency than previously reported (Wilson et al., 1979; M. Kiefer, R. Fraley, D. Papahadjopoulos, and G. Bruening, unpublished experiments; R. Fraley, D. Dellaporta, M. Gordon, E. Nester, and D. Papahadjopoulos, unpublished experiments) and in the case of DNA has been used to transform cell types which do not respond to other techniques commonly used for introducing DNA into cells (S. Dellaporta, K. Giles, R. Fraley, D. Papahadjopoulos, A. Powell, M. Thomashow, G. Nester, and P. Gordon, unpublished experiments). However, it should be emphasized that further improvements are necessary before this method can be used routinely to transform or to transfect mammalian cells. In the present study, we have extended our initial observations on liposome-mediated delivery of SV40 DNA to African green monkey kidney (AGMK) cells (Fraley et al., 1980) and have examined the interaction of a variety of liposome preparations with these cells. In addition, we report on methods which increase the effective intracellular delivery of both high and low molecular weight compounds entrapped in liposomes and present evidence bearing on the mechanism of uptake.

Experimental Procedures

Lipid and Other Materials. The sources and purity of the phospholipids used in this study have been reported previously (Mayhew et al., 1979). All lipids were stored in chloroform in sealed ampules under argon at −70 °C until use. [^{3}H]-Poly(A) (25–89 Ci/mmol) and [^{3}H]inulin (>300 mCi/mmol) were purchased from Amersham. [^{14}C]Sucrose (~600 Ci/mmol) and [^{3}H]dipalmitoylphosphatidylcholine (DPPC) (~100 mCi/mmol) were obtained from New England Nuclear. [^{3}H]DPPC was further purified on a silicic acid column and its purity verified by thin-layer chromatography. Stearylamine (SA), agarose (type II), cytochalasin B, chloroquine, colchicine, sodium azide, and 2-deoxyglucose were purchased from Sigma.

Preparation of Phospholipid Vesicles. Purified SV40 DNA (1–100 μg) or carboxyfluorescein (CF) (72 mM) was encapsulated in large unilamellar vesicles (LUV) by the reverse-phase evaporation (REV) method of Szoka & Papahadjopoulos (1978). The procedure for entrapping DNA employed modifications described previously (Fraley et al., 1980) for efficient encapsulation of small aqueous volumes (50–350 μL). A 0.1-μCi sample of [^{3}H]poly(A) or ^{3}H-labeled pBR322 DNA (gift from Dr. L. Rall) was added to the DNA preencapsulation mixture to allow quantitation of encapsulated SV40. Both radioactive tracers were trapped with equal efficiency. Multilamellar PS vesicles for electron microscopy studies were prepared as described (Olson et al., 1979).

All vesicles were extruded through polycarbonate membranes of controlled pore diameter (Olson et al., 1979; Szoka et al., 1980) to ensure homogeneity; MLV were sized to 1.0 μm and LUV to 0.4 μm. Unencapsulated carboxyfluorescein was separated from liposomes by gel filtration (Sephadex G-75), and unencapsulated DNA was removed by floatation of liposomes on Ficoll gradients (Fraley et al., 1980). The vesicle-containing fraction was analyzed for lipid phosphate (Bartlett, 1959), and DNA encapsulation was determined by radioactivity. SV40 DNA has been extracted from vesicles (Bligh & Dyer, 1959; 2-propanol substituted for methanol) and checked by plaque assay for biological activity (Fraley et al., 1980) or by agarose gel electrophoresis for nicking of the supercoiled form. The results show no significant loss of infectivity and no apparent degradation with the REV encapsulation procedure.

Cell Culture and Plaque Assays. CV-1P, an established cell line of African green monkey kidney (AGMK) cells, was cultured as described by Mertz & Berg (1974) by using Dulbecco's-modified Eagle's medium supplemented with 5% bovine newborn serum. Viral DNA was obtained from CV-1 cells infected at low multiplicity with wild-type SV40 virus. The viral DNA was extracted and purified by CsCl–ethidium bromide centrifugation as described previously (Hirt, 1967).

Plaque assays using purified viral DNA were performed as described by Mertz & Berg (1974). The CV-1P monolayers were washed twice with Tris-buffered saline (TBS; Kimura & Dulbecco, 1972) and incubated for 15.0 min at room temperature with 0.2 mL of TBS containing DNA (0.01–0.1 ng) and 500 μg/mL DEAE-dextran. After the monolayers were washed twice more with TBS, they were overlaid with agar medium as previously described (Mertz & Berg, 1974). The infectivity of the SV40 DNA in the presence of DEAE-dextran was typically 5×10^6 pfu/μg of DNA, and the reproducibility of this value was within 30% from experiment to experiment.

Liposome–Cell Incubations. Incubation of cells with liposome-encapsulated SV40 DNA was carried out as described previously (Fraley et al., 1980). TBS (0.2 mL) containing between 0.1 and 10 ng of encapsulated SV40 DNA and 0.5–500 nmol of phospholipid was added to the washed cell monolayers, and the plates were incubated for 30 min at 37 °C. Following the incubation, the monolayers were overlaid with 1.0 mL of TBS containing 25% (v/v) glycerol for 4 min at 25 °C. (It was subsequently found that incubation at 37 °C increased infectivity 2–4-fold). The monolayers were then washed twice with TBS and overlaid with agar medium as described above. The competition experiments described in Figure 5 were performed in a similar manner except that buffer-loaded ("empty") vesicles (5–500 nmol of lipid) were premixed with SV40 DNA containing vesicles or with radioactively labeled vesicles (5 nmol of lipid) prior to their incubation with cells.

The effects of various drugs and inhibitors on liposome-mediated SV40 DNA delivery were determined according to the above protocol except that CV-1P cells were exposed to the appropriate drug both prior to and during exposure to liposomes. The details of the various incubations are given in the legend for Table II. The effect of such treatments on the rate of endocytosis in CV-1P cells was determined by monitoring the uptake of [^{14}C]sucrose. TBS (0.2 mL) containing [^{14}C]sucrose (150 μCi/mL) and 4 mM unlabeled sucrose was added to washed cell monolayers and incubated for 30 min at 37 °C. The cells were then washed twice with isotonic TBS containing 80 mM unlabeled sucrose, twice with TBS, removed from the tissue culture plate by trypsinization (0.25% trypsin in saline with 0.1% EDTA for 5 min at 37 °C), and transferred to scintillation vials for determination of radioactivity. The rate of RNA synthesis was determined by monitoring [^{3}H]uridine incorporation into Cl_3CCOOH-precipitable material and was not depressed in drug-treated cells 24–36 h after incubation with liposomes.

Liposome–Cell Association Studies. Liposomes used for cell binding studies were prepared as described above except that either [^{3}H]DPPC (50 μCi) or [^{3}H]inulin (50 μCi) was included in the preparations as a radioactive label for vesicle

Table I: Correlation between Vesicle Binding, Leakage, and Infectivity of SV40 DNA Delivery by Various Liposome Preparations[a]

vesicle preparation[b]	encapsulation efficiency[c] (μg of DNA/μmol of lipid)	cell-associated lipid[d] (nmol bound)	vesicle contents[e] (% retained)	efficiency of delivery[f] (pfu/μg of DNA)	
				−glycerol	+glycerol
PC	0.31	0.51	28	1.3×10^2	7.2×10^2
PC-SA	0.44	0.57	87	0.5×10^2	1.7×10^2
PS	0.44	2.7	43	1.8×10^3	6.5×10^4
PG	0.36	2.9	8.8	1.1×10^2	6×10^3
PS-Chol	0.39	2.5	84.7	2.8×10^3	1.2×10^5
PG-Chol	0.42	2.6	49.2	1.6×10^3	6.8×10^4

[a] Liposomes were prepared by the reverse-phase evaporation technique starting with 10 μmol of phospholipid and 10 μg of SV40 DNA. The vesicle preparations were sized and sterilized by passage through a 0.4 μm Unipore filter, and the vesicles were separated from unencapsulated DNA by flotation on Ficoll gradients. Extraction of the encapsulated SV40 DNA and analysis on agarose gels revealed no degradation of the DNA. Liposome incubations with AGMK cells and glycerol treatment were performed as described under Experimental Procedures. Plaque assays were carried out with 0.1, 1, or 10 ng/plate of SV40 DNA encapsulated in the different vesicle preparations. The extent of cell-induced leakage of vesicle contents was determined by comparing the amounts of cell-associated vesicle lipid and encapsulated contents for each vesicle preparation at 10 nmol of lipid added/plate. [b] All preparations encapsulated between 0.3 and 0.5 μg of SV40 DNA/μmol of phospholipid. [c] The amount of encapsulated DNA per micromole of phospholipid. [d] Amount of cell-associated lipid measured after incubation of 10 nmol of vesicle lipid/plate. [e] Determined from the amount of cell-associated vesicle contents ([^{3}H]inulin) measured for each preparation at 10 nmol of vesicle lipid added/plate. [f] The infectivity of liposome-encapsulated SV40 DNA in glycerol-treated (25% v/v) and nontreated cells was determined by plaque assays.

lipid or aqueous interior, respectively. Incubations of radioactive liposomes with AGMK cells were performed as described above. The cell monolayers were subsequently washed 3 times with 3 mL of TBS, removed from the tissue culture plate by trypsinization, and transferred to scintillation vials for determination of cell-associated radioactivity. Control experiments were performed to measure the uptake of free [^{3}H]inulin by cells under these incubation conditions and to determine the extent to which [^{3}H]DPPC-labeled vesicles bind to serum-coated plates in the absence of cells. All results are adjusted for such backgrounds, although even at the lowest vesicle concentrations (5 nmol of lipid) these corrections were less than 5% of the experimental values.

Microscopy. Fluorescence photographs were taken on Tri X (Kodak) film with a Zeiss microscope equipped with fluorescence optics and epillumination. For electron microscopy, control of liposome-treated cells were rinsed twice with TBS and were fixed sequentially with 2.5% glutaraldehyde (1 h, 37 °C), 6% acrolein (2 h, 22 °C), and 3% $KMnO_4$ (2 h, 22 °C) in 0.2 M sodium cacodylate buffer, pH 7.4. Disks of these in situ fixed cells were punched out of the culture dish, dehydrated in ethanol, dried in a Bomar critical point drying apparatus using CO_2, and sputter-coated with gold to a thickness of 200 Å. After examination in a Cambridge 150 scanning electron microscope, the disks were removed from the specimen stubs, and the cells were infiltrated with two changes of *n*-butyl glycidal ether, an epoxide which is miscible in Quetol 651. Subsequent embedment in Quetol 651 was accomplished by using the Kushida (1974) method. After ultrathin sections were cut perpendicular to the plane of cell growth, they were poststained with lead citrate and uranyl acetate and examined in a Phillips 300 transmission electron microscope operating at 60 kV.

Results

Effect of Vesicle Lipid Composition and Glycerol Treatment on the Delivery of SV40 DNA to Cells. We have shown previously that following the initial incubation of liposomes with cells, exposure to glycerol solutions can enhance substantially (up to 200-fold) liposome-mediated DNA delivery. It is apparent from the data in Table I and Figure 1 that the glycerol treatment is most effective in enhancing SV40 DNA delivery by negatively charged liposomes (PS and PG); such vesicles demonstrate a 40–60-fold increase in infectivity following exposure to glycerol (25% v/v). In contrast, neutral

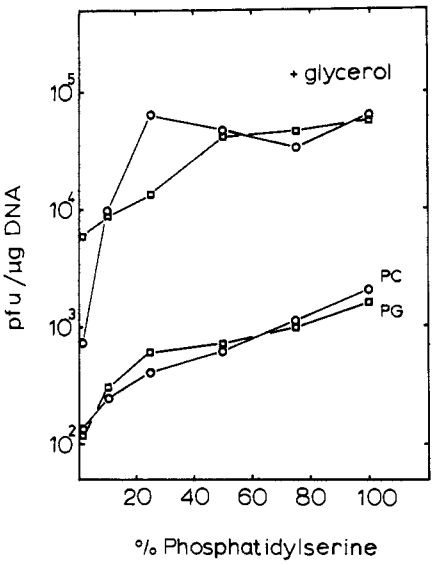

FIGURE 1: Effect of phosphatidylserine on liposome-mediated SV40 DNA delivery to AGMK cells. Liposomes were prepared as described in the legend to Table I. Cell incubations and treatment with glycerol (25% v/v) were performed as described under Experimental Procedures. Infectivity of SV40 DNA (0.1–10 ng added/plate) encapsulated in vesicles composed of a mixture of PS and PC (○) and PS and PG (□).

(PC) or positively charged (PC–SA) liposomes show only a 3–6-fold increase in infectivity following treatment with glycerol. Inclusion of PS in either PG or PC vesicles markedly enhances infectivity, both with and without glycerol treatment (Figure 1), and increasing the percentage of PS results in a progressive increase in infectivity. It should be noted that the relative encapsulation of SV40 DNA in these various liposome preparations is similar (Table I), so that the large differences in infectivity must reflect other differences, such as the extent of cell binding, vesicle leakage, or the efficiency of intracellular DNA delivery. Such parameters are examined in the following section.

Association of Vesicle Lipid and Internal Contents with Cells. In order to determine whether the large differences in infectivity of SV40 DNA encapsulated in liposomes of various compositions (Table I and Figure 1) reflect differences in the extent of vesicle binding or leakage of encapsulated DNA, we have examined the correlation between cell-associated vesicle lipid and vesicle contents as a function of liposome composition

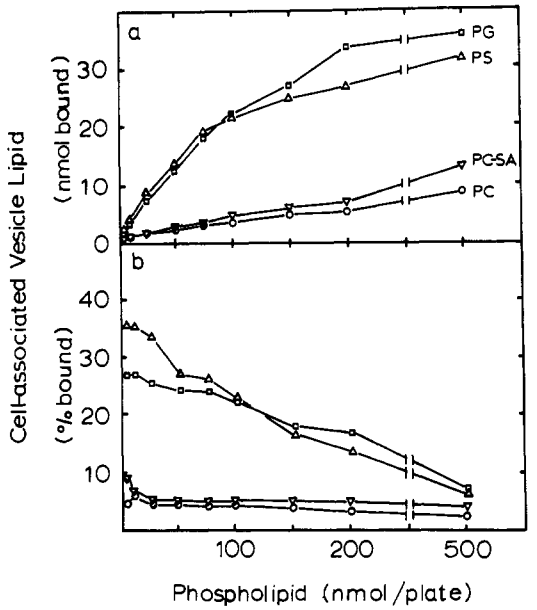

FIGURE 2: Association of vesicle lipid with AGMK cells following incubation with various liposome preparations. Liposomes, containing [^{3}H]DPPC (5 μCi/μmol of vesicle phospholipid), were prepared and incubated with cells (5–500 nmol of lipid added/plate) under conditions identical with those used for liposome-mediated DNA transfection (Table I). The cell monolayers were washed extensively, and the amount of cell-associated vesicle lipid was determined as described under Experimental Procedures. Postincubation treatment of cells with glycerol (25% v/v) did not influence the amount of cell-associated vesicle lipid (not shown). (a) Nanomoles of cell-associated vesicle lipid following incubation with increasing amounts of radioactively labeled PS (△), PG (□), PC (○), and PC–SA (▽) vesicles; (b) percentage of added vesicle lipid that is cell associated [symbols same as in (a)].

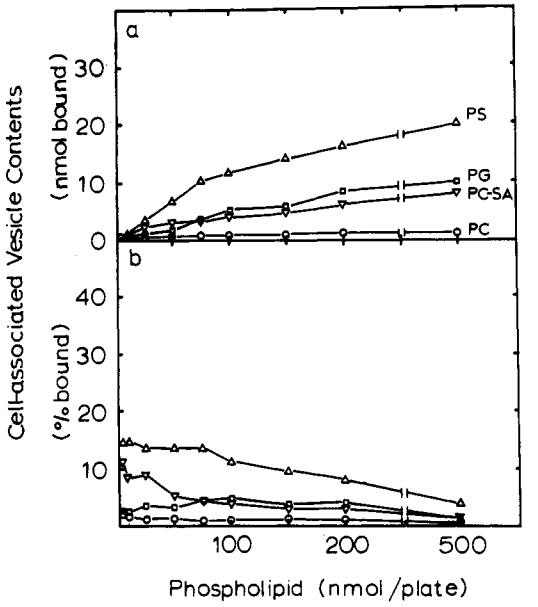

FIGURE 3: Association of vesicle contents with AGMK cells following incubation with various liposome preparations. Liposomes, containing [^{3}H]inulin (0.15 μCi/μL) as a marker for internal contents, were prepared and incubated with cells (5–500 nmol of lipid added/plate) under conditions identical with those used in Figure 2. The cell monolayers were washed extensively, and cell-associated radioactivity was determined as described under Experimental Procedures. The amount of cell-associated vesicle contents is expressed as vesicle lipid equivalents to permit direct comparison with the values determined for cell-associated vesicle lipid in Figure 2. Postincubation treatment of cells with glycerol (25% v/v) reduced the amount of cell-associated vesicle contents by approximately 25% in all incubations (not shown). (a) Cell-associated vesicle contents (nanomoles of vesicle lipid equivalents) following incubation with increasing amounts of radioactively labeled PS (△), PG (□), PC (○), and PC–SA (▽) vesicles; (b) percentage of added vesicle contents that is cell associated [symbols same as in (a)].

(Figures 2 and 3). Vesicles of the same composition as those used in Table I, and containing [^{3}H]DPPC as a trace radioactive phospholipid label, were incubated with cells under the same conditions as those used for liposome-mediated DNA transfection; the amount of cell-associated vesicle lipid was determined after extensive washing of the cell monolayer. It should be emphasized that this experimental approach does not distinguish between liposomes adsorbed to the cell surface and those taken up by the cell by fusion, endocytosis, or some other process; therefore, these vesicles are referred to as cell associated. It is unlikely under our incubation conditions (37 °C and serum-free buffer) that the extent of cell–vesicle association may be overestimated as a result of the exchange of [^{3}H]DPPC from liposomes to the plasma membrane, since exchange occurs as a predominant process only at low temperatures (Struck & Pagano, 1980).

The amount of cell-associated lipid is greater after incubations with negatively charged PS and PG vesicles (Figure 2a, approximately 30 nmol/5 × 10^6 cells) than with PC or PC–SA vesicles (approximately 10 nmol/5 × 10^6 cells). This difference is accentuated greatly at lower vesicle concentrations (≤50 nmol added) where the association of negatively charged liposomes was approximately 5–9 times greater than that observed for either the PC or the PC–SA vesicles (Figure 2). As the amount of lipid used in DNA delivery experiments generally is less than 50 nmol/plate, it is clear that the affinity of liposomes for cells is a major parameter in determining the infectivity of various lipid compositions.

The large difference observed between the amount of cell-associated PS and PC or PC–SA vesicles can account for the relatively low efficiency of DNA delivery by the latter preparations and also suggests that including PS in mixed PS–PC vesicles (Figure 1) may increase the efficiency of DNA delivery simply by promoting enhanced vesicle uptake by cells. However, differences in the number of cell-associated vesicles cannot account for the low infectivity of SV40 DNA encapsulated in liposomes of another negatively charged phospholipid, PG (Table I, Figure 1). Leakage of vesicle contents could reduce dramatically the amount of vesicle-encapsulated material (DNA) that is available for intracellular delivery. Liposomes containing [^{3}H]inulin were used (Figure 3a) to determine the association of vesicle contents with cells under incubation conditions identical with those in Figure 2. The use of inulin as a marker for vesicle internal contents is preferable to using a radioactively labeled DNA because it eliminates problems associated with cell binding or uptake of free DNA (or oligonucleotides) which may have leaked from liposomes during the incubation. The amount of [^{3}H]inulin (expressed as vesicle lipid equivalents) associated with cells is highest for PS vesicles, while the association of PG liposome contents with cells is much lower (approximately 5-fold; Figure 3b and Table I). PC vesicles leak approximately 75% of their contents upon interactions with cells (Table I). In contrast, the association of PC–SA vesicle contents is nearly equivalent to the amount of cell-associated lipid, indicating that positively charged vesicles leak very little when exposed to cells.

These results suggest that while the negatively charged PS and PG vesicles associate with cells to similar degrees (Figure 2), the greater leakiness of PG vesicles decreases the intracellular delivery of contents. This interpretation is strengthened by the observation that including 50 mol % cholesterol (Chol) in PG vesicles, which reduces cell-induced leakage approximately 5-fold, also enhances markedly (approximately

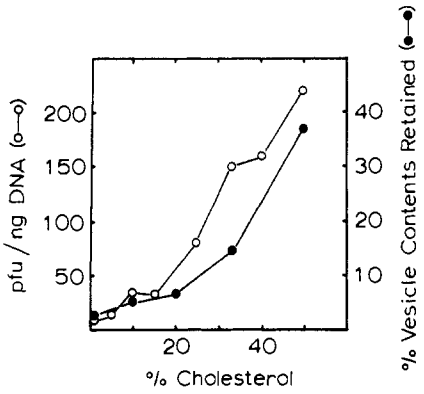

FIGURE 4: Effect of cholesterol on the leakage and delivery of SV40 DNA by PG liposomes. Liposomes composed of PG and the mole percent of cholesterol shown in the figure, and containing either [^{3}H]inulin or SV40 DNA, were prepared as described under Experimental Procedures. The amount of cell-associated vesicle contents following the glycerol treatment were determined as in Figure 2, and the extent of cell-induced leakage was determined as in Table I. Plaque assays were performed as described under Experimental Procedures. A correlation between SV40 DNA infectivity and vesicle leakage also was observed in the absence of the glycerol treatment (data not shown). Infectivity of SV40 DNA encapsulated in vesicles composed of mixtures of PG and cholesterol following glycerol treatment (○). Retention of PG vesicle contents in the presence of increasing amounts of cholesterol (●).

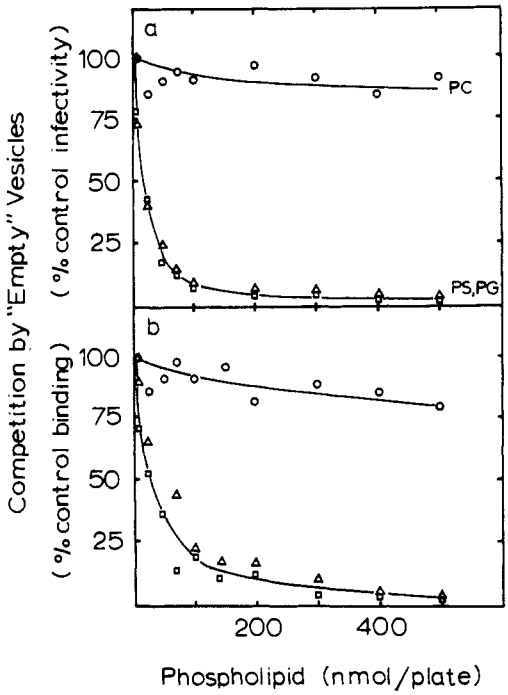

FIGURE 5: Competition for PS vesicle binding and SV40 DNA delivery to AGMK cells by empty vesicles. PS vesicles (5 nmol of lipid) containing either SV40 DNA (prepared as described in Table I) or [^{3}H]DPPC (prepared as described in Figure 2) were mixed with increasing amounts (5–500 nmol of lipid) of empty or unlabeled liposomes prior to incubation with cells. The extent of PS vesicle binding or liposome-mediated SV40 DNA delivery was determined as described under Experimental Procedures and is expressed as the percent of the value obtained in the absence of competing liposomes. (a) Infectivity of SV40 DNA encapsulated in PS vesicles in the presence of increasing amounts of empty PS (△), PG (□), and PC (○) vesicles; (b) percentage of PS vesicle lipid that becomes cell associated in the presence of increasing amounts of unlabeled PS, PG, and PC vesicles [symbols same as in (a)].

10-fold) the delivery of SV40 DNA (Table I). Similarly, increasing amounts of PS in mixed PS–PG vesicles can enhance DNA delivery (Figure 1) by increasing retention of vesicle contents during cell–vesicle interaction.

The effect of cholesterol on reducing the extent of cell-induced vesicle leakage, and its correlation with increased intracellular delivery of liposome contents, is shown more clearly in Figure 4. A significant reduction in the leakage from PG vesicles and an increase in SV40 DNA infectivity are observed when 20 mol % cholesterol is included in the liposome preparation. Increasing the amount of cholesterol to 50 mol % further enhances delivery. The data in Table I show that PS vesicles retain a high percentage of their contents (43%) upon cell binding and that including 50 mol % cholesterol increases this value to 85%; this 2-fold decrease in leakage correlates well with the approximate 2-fold increase in DNA delivery (Table I). Inclusion of 50 mol % cholesterol in either PC or PC–SA vesicles does not enhance DNA delivery (data not shown).

Competition Experiments with Unlabeled or Empty Liposomes. The apparent discrepancy between the amount of cell-associated PC–SA vesicle contents and their low efficiency of delivery (with or without glycerol) is not clear, but it is reasonable to speculate that positively charged and neutral liposomes bind to different sites on the cell surface than do negatively charged liposomes and that such sites do not promote efficient vesicle uptake. This possibility was examined in greater detail in Figure 5 by determining the ability of different liposome preparations to compete with PS vesicles for cell binding or DNA delivery. In these experiments, PS vesicles (5 nmol of lipid), either labeled with [^{3}H]DPPC or containing SV40 DNA, were premixed with increasing amounts (5–500 nmol of lipid) of unlabeled PS, PG, or PC liposomes prior to their incubation with AGMK cells. As expected from the lipid association studies (Figure 2), PS and PG vesicles complete identically with radioactively labeled PS liposomes (Figure 5), and both preparations decrease the amount of cell-associated radioactivity. The infectivity of SV40 DNA encapsulated in PS vesicles was also reduced in the presence of increasing amounts of empty PS and PG vesicles (Figure 5), providing additional evidence for the correlation between the extent of vesicle binding and the amount of intracellular delivery. Neutral (PC) vesicles were ineffective in reducing PS vesicle binding and DNA delivery even when added in 100-fold molar excess. This observation suggests that neutral liposomes may have different binding sites on the cell surface or that negative liposomes bind to additional sites not available to PC. While we were unable to perform similar experiments with positively charged PC–SA vesicles because they aggregated with PS vesicles, it seems reasonable to suppose that positively charged liposomes may also bind to different sites.

Glycerol Stimulates Uptake of Liposome Contents by an Energy-Dependent Mechanism. Polyalcohols are known to alter membrane structure (Maroudas, 1975) and hydration properties (Tilcock & Fisher, 1979) as well as induce membrane fusion (Maggio et al., 1976) and endocytosis (Norberg, 1970). Therefore, it seems likely that glycerol may stimulate the cellular uptake of liposomes by increasing the frequency of fusion or endocytosis.

Examination of control AGMK cultures by scanning electron microscopy (SEM) (Figure 6A) shows closely apposed cells having apical surfaces populated with short, uniform microvilli. The cells are united by villous ridges typical of contact-inhibited epithelial cells. In liposome-treated cells (Figure 6B), PS vesicles are identified as single or clustered spherical particles predominantly located between cells. In contrast, glycerol-treated cells (Figure 6C) appear to have highly ruffled surfaces and are more rounded than control cells, possibly indicating an alteration in the cytoskeletal structure.

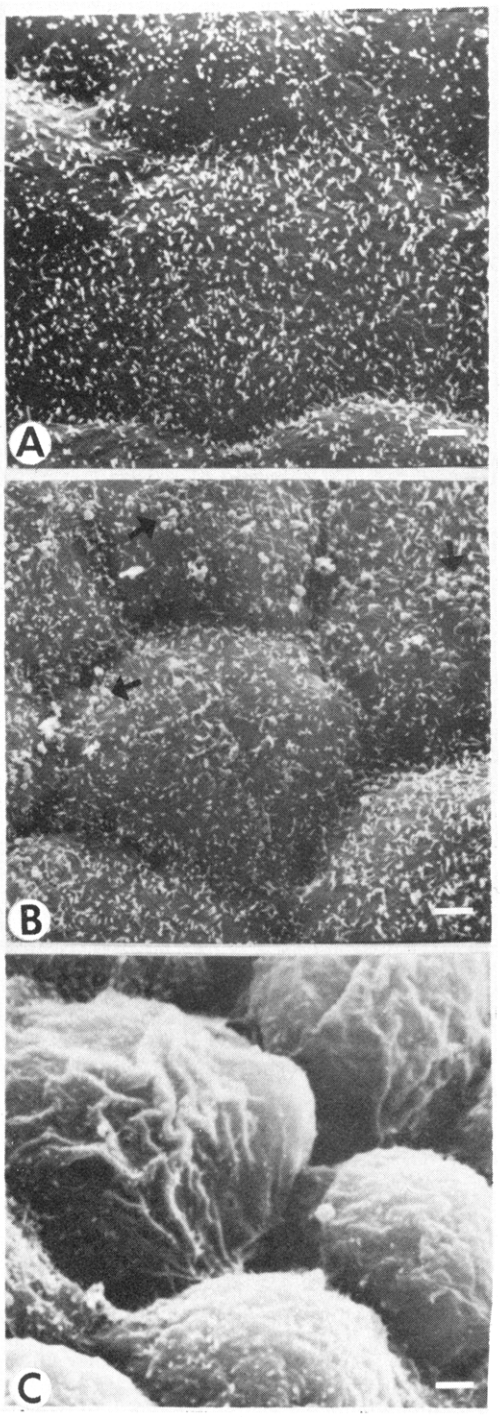

FIGURE 6: Effect of glycerol treatment on cell morphology. Multilamellar PS liposomes, sized to 1.0 μm by extrusion through polycarbonate filters (Olson et al., 1979), were used in the electron microscopy studies to ensure adequate resolution. Cell incubations, treatment with glycerol (25% v/v), and conditions for microscopy were as described under Experimental Procedures. Scanning electron micrographs of control AGMK cells show tightly apposed cells with numerous short uniform microvilli (A). Following incubation with 500 nmol of PS vesicles, cells display solitary vesicles and clusters of vesicles (arrows) located among the microvilli (b); cell treated with glycerol (20%) after exposure to PS vesicles show numerous ruffles, scant microvilli, and few vesicles (C). (Marker =2 μm.)

Only an occasional liposome can be seen on the surface of such cells. Since the amount of vesicle lipid remains constant before and after exposure to glycerol (data not shown), this observation suggests that a large fraction of the vesicles have been internalized by the cells. In ultrathin sections of glycerol-treated cells, liposomes appear to be clustered together in large

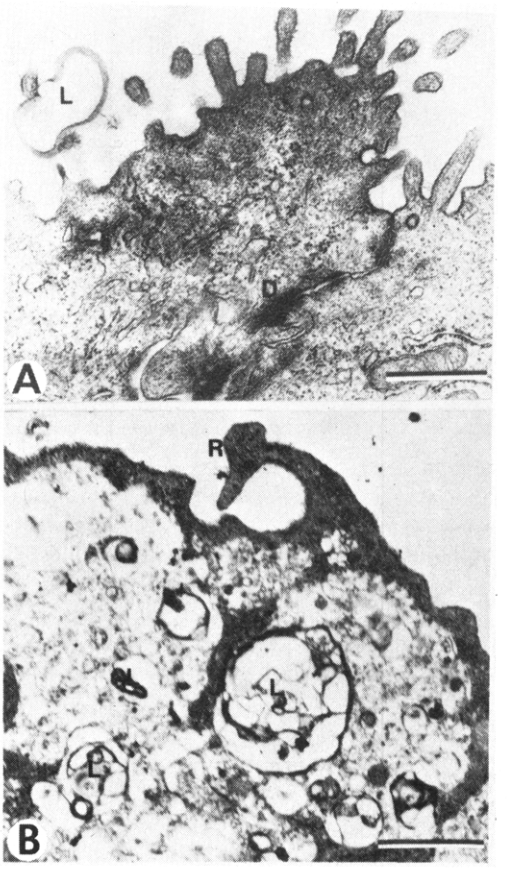

FIGURE 7: Effect of glycerol treatment on cell structure. Liposome preparation and incubation conditions were as described in the legend to Figure 6. Sample preparation for electron microscopy was as described under Experimental Procedures. Transmission electron micrograph of a cell incubated with PS vesicles shows a 1-μm liposome (marked L) attached to the microvillous ridge between two cells and a junctional complex (D) typical of epithelial cells. (A) Glycerol-treated cell (plus PS vesicles) displays a ruffle (marked R) enclosing a macropinocytotic space, and multilamellar vesicles (L) within membrane-bound inclusions and surrounded by a highly vacuolated cytoplasm (B). (Marker = 1 μm.)

vacuolar areas within the cell (Figure 7B). Ultrathin sections of control (not shown) and PS-treated cultures (Figure 7A) do not show such vacuolization but reveal a well-developed actin network beneath a villous plasma membrane; a PS liposome seems attached to the cell surface at the microvillous ridge. The submembranous actin network appears more condensed and less organized in glycerol-treated cells than in the control or PS-treated cells.

The membrane ruffling and extensive vacuolization associated with glycerol-treated cells (Figure 6C) are often observed in cells which are actively endocytotic (Brunk et al., 1976). Therefore, it seems possible that glycerol stimulates the cellular uptake of liposomes by endocytosis. In order to provide additional insight into the mechanism of glycerol enhancement of liposome delivery, various agents which are known to affect endocytosis, microtubule organization, or lysosome function were tested for their ability to alter liposome delivery (as measured by SV40 DNA infection) and endocytosis (as measured by sucrose uptake) in AGMK cells. In agreement with a number of recent investigations with mammalian cells (Silverstein et al., 1977, 1978), the rate of endocytosis was decreased approximately 90% in the combined presence of sodium azide and 2-deoxyglucose (although neither drug alone was as effective) but was not affected or only partially reduced in the presence of colchicine, cytochalasin B, or chloroquine (Table II).

Table II: Effect of Various Drugs and Inhibitors on Endocytosis and Liposome-Mediated SV40 DNA Delivery in AGMK Cells[a]

incubation conditions[b]	[^{14}C]sucrose uptake[c] (% of control)	SV40 DNA infectivity[d] (% of control)	
		−glycerol	+glycerol
control (no inhibitors)	100	100	100
sodium azide/2-deoxy-glucose	5	69	11
cytochalasin B	46	66	125
colchicine	81	145	11
chloroquine	87	89	367
chloroquine[e]			762

[a] SV40 DNA delivery by liposomes and the accumulation of [^{14}C]sucrose by cells were assayed as described under Experimental Procedures. The length of pretreatment and the concentration of the drugs and inhibitors used in the table were either the optimum determined for inhibition or equivalent to those previously reported in the literature. The results shown are the average of three separate experiments and are expressed as the percent of the value obtained in the absence of inhibitors. The rate of endocytosis measured for control AGMK cells under these incubation conditions was approximately 0.36 μL (10^6 cells)$^{-1}$ h^{-1}; this value is in close agreement with those previously reported for mammalian cells (Silverstein et al., 1977). RNA synthesis was measured by [^{3}H]uridine incorporation into acid-precipitable radioactivity 36 h after removal of the inhibitors. In no case was uridine incorporation depressed relative to untreated control cells (data not shown). [b] The length of pretreatments and concentrations of inhibitors used were the following: 5 mM sodium azide/50 mM 2-deoxyglucose for 0.5 h, 20 μg/mL cytochalasin B for 1 h, 100 μM colchicine for 2 h, and 100 μM chloroquine for 1 h. All drugs were also present during the incubation with vesicles and glycerol treatment. [c] The rate of [^{3}H]sucrose uptake in control cells was 14 900 cpm (5×10^6 cells)$^{-1}$ (0.5 h)$^{-1}$ or 0.36 μL (10^6 cells)$^{-1}$ h^{-1}. Values shown are averages of three experiments (in duplicate), and the variability was within 15%. [d] The control values for infectivity of SV40 DNA encapsulated in PS vesicles were 2×10^3 and 2.6×10^5 pfu/μg of DNA in the absence and presence of the glycerol treatment, respectively. Values shown are averages of three to five experiments (each in duplicate). [e] Vesicles were diluted in serum-free Dulbecco's minimum Eagle's medium for incubation with cells, and the incubation with glycerol was continued for 30 min.

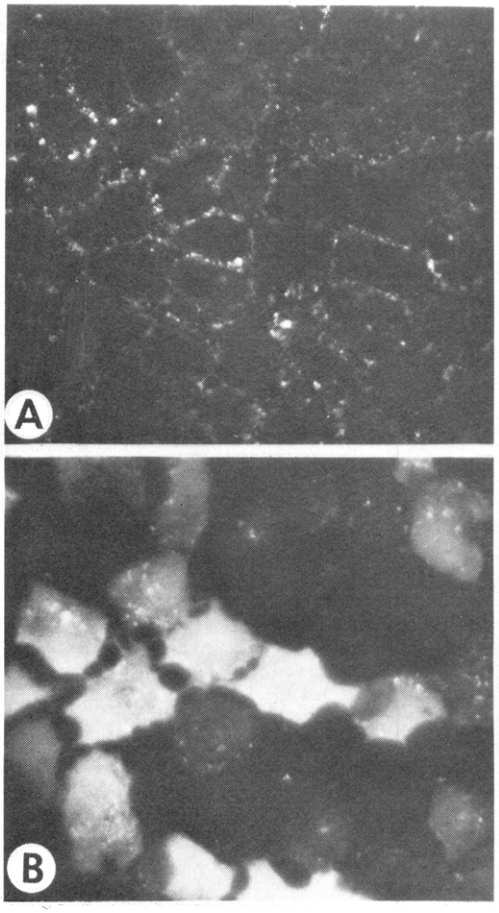

FIGURE 8: Effect of glycerol treatment on the delivery of liposome-encapsulated carboxyfluorescein to AGMK cells. PS–Chol liposomes containing carboxyfluorescein were prepared as discussed under Experimental Procedures, except that carboxyfluorescein (75 mM) was substituted for NaCl (150 mM). Fluorescence micrographs of AGMK monolayers following incubation with 500 nmol of PS–Chol vesicles containing carboxyfluorescein; (A) control cells; (B) glycerol-treated cells.

The effects of the inhibitors on liposome-mediated DNA delivery in glycerol-treated cells paralleled those obtained for sucrose uptake (endocytosis) in all cases but one; chloroquine treatment enhanced DNA infectivity (approximately 4-fold). In the absence of glycerol, liposome-mediated DNA delivery was not enhanced by chloroquine under the present experimental conditions, and infectivity was only moderately (approximately 30%) reduced in the presence of sodium azide and 2-deoxyglucose. A nonspecific effect of the various inhibitors on cell metabolism and SV40 DNA expression is unlikely since the infectivity of DNA by the DEAE-dextran method was unchanged and the rate of RNA synthesis measured in the inhibitor-treated cells 24–36 h after incubation with liposomes was found to be comparable to that of control cells (data not shown).

Optimal Delivery of Liposomal Contents to Cells. The results in Table II indicate that glycerol stimulation of liposome delivery is energy dependent. This is supported by the observation that infectivity can be increased (2–4-fold) if all incubations are performed in serum-free growth media. The presence of serum reduces the efficiency of delivery, presumably due to its deleterious effect on liposome integrity (Mayhew et al., 1979; Gregoriadis & Davis, 1979; Allen & Cleland, 1980). Infectivity can be increased further by extending the glycerol treatment for 30 min. Combining these incubation conditions with the chloroquine (100 μM) pretreatment results in values for liposome-mediated infectivity of 2×10^6 pfu/μg of DNA (Table II). This represents a 1000-fold improvement in infectivity over simply incubating AGMK cells with liposomes and compares favorably with the calcium phosphate (0.1×10^6 pfu/μg of DNA) and DEAE-dextran (5×10^6 pfu/μg of DNA) methods for introducing DNA into AGMK cells (data not shown).

In order to determine if these conditions (PS–Chol liposomes, glycerol treatment, etc.) are also effective in increasing the cellular uptake of other liposome-encapsulated molecules, the delivery of the entrapped fluorescent dye, carboxyfluorescein (CF), was examined. Following incubation of AGMK cells with the dye encapsulated in PS–Chol liposomes, intracellular fluorescence is quite low, and the majority of the fluorescence is localized near the cell periphery. The majority of cell-associated liposomes have not delivered their contents intracellularly but remain adsorbed to the cell surface (Figure 8A). However, subsequent treatment with glycerol (Figure 8B) dramatically increases the intracellular fluorescence. In agreement with the results for SV40 DNA delivery, AGMK cells that have been pretreated with azide and 2-deoxyglucose show no increase in intracellular fluorescence following exposure to glycerol (unpublished observations). Glycerol does not enhance the cellular uptake of free carboxyfluorescein (10 mM; data not shown), indicating that the treatment is not simply increasing cell membrane permeability. Also in agreement with the results obtained for SV40 DNA delivery,

incubation of cells with PC or PC–SA liposomes containing carboxyfluorescein results in no detectable intracellular fluorescene, with or without the glycerol treatment (data not shown).

Discusssion

Apart from the obvious genetic implications of liposome-mediated DNA delivery, the expression of foreign DNA in cells provides an unequivocal and sensitive biological assay for monitoring the intracellular delivery of liposome contents. Since only those DNA molecules which escape lysosomal degradation, reach the nucleus, and are expressed will score as a positive delivery event, it is likely that the total extent of liposome delivery to cells is underestimated by this method. Nevertheless, the existence of such an assay, together with recent methodological advances permitting the efficient encapsulation of large molecules, allows for the systematic determination of those lipid compositions, incubation conditions, and cell treatments which favor the increased intracellular delivery of liposome contents.

The results presented in Table I indicate that liposomes containing PS are the most efficient carriers for introducing SV40 DNA into AGMK cells, and furthermore, the extent of delivery is shown to be proportional to the percentage of PS present in the vesicle preparation (Figure 1). The high efficiency of intracellular delivery promoted by PS vesicles can be understood in terms of their high affinity for cells (Figure 2) and their comparatively low rate of leakage of contents upon incubation with cells (Figure 3). A number of investigations of liposome–cell interactions have reported that negatively charged liposomes are taken up only 2–3-fold more avidly than are neutral liposomes (Szoka et al., 1979). It should be noted that such studies were conducted with amounts of lipid which saturate the liposome-binding capacity of cells (>100 nmol added/10^6 cells) so that the large differences in binding which are apparent at lower lipid concentrations (≤50 nmol added/10^6 cells) have been previously overlooked.

Several earlier reports demonstrated a greater binding to cells by positively charged liposomes (Szoka et al., 1980; Magee & Miller, 1972; Magee et al., 1976; Batzri & Korn, 1975). However, all these studies employed cells growing in suspension culture, while our studies and others which reported higher or equivalent binding by negatively charged liposomes (Hoekstra & Scherphof, 1979; Papahadjopoulos et al., 1974; Huang & Pagano, 1975) have utilized monolayer cells. We have observed that when binding experiments are performed with AGMK cells in suspension, the level of binding by PS vesicles remains unchanged (approximately 30 nmol of lipid/5 × 10^6 cells), while the apparent binding by PC–SA vesicles increases 2–3-fold (20–35 nmol of lipid/5 × 10^6 cells) and the cells become extensively aggregated (unpublished observation).

Inclusion of cholesterol in PG vesicles markedly enhances (10-fold) infectivity concomitant with reducing leakage of aqueous contents (Table I and Figure 4). This provides strong evidence that in addition to vesicle binding, the extent of cell-mediated leakage of encapsulated materials can influence dramatically the efficiency of intracellular delivery. A number of recent reports have indicated that the inclusion of cholesterol reduces the serum-induced leakage of liposome contents (Mayhew et al., 1979; Gregoriadis & Davis, 1979; Allen & Cleland, 1980) and increases the clearance time of intact liposomes in the circulation as well as their efficacy as drug carriers (Mayhew et al., 1979). The results in Table I extend these observations by demonstrating for the first time that cholesterol can also reduce the extent of cell-induced vesicle leakage in vitro and as a result, increase the amount of encapsulated material which can be delivered intracellularly.

The high affinity of negatively charged vesicles for cells (Figure 2), the selective enhancement of the infectivity of negatively charged liposomes by glycerol treatment (Table I), and the failure of neutral (PC) vesicles to compete for binding or delivery by PS vesicles (Figure 5) suggest that negatively charged liposomes may bind to specific sites on the cell surface, facilitating uptake by cells. Supporting this possibility are the results of Martin & Lagunoff (1980) indicating that PC vesicles, which cannot stimulate histamine release from mast cells, also cannot compete with the binding of PS vesicles to purified mast cells; this has been interpreted as indicating a specific interaction between PS vesicles and mast cells. In a more general sense, it should be noted that Goldstein et al. (1979) have proposed the existence of binding sites on a variety of cell types which recognize macromolecules with multiple negative charges.

Glycerol enhancement of the infectivity of SV40 DNA encapsulated in negatively charged liposomes is not well understood; however, an increase in surface membrane activity is suggested by the extensive vacuolization and membrane ruffling observed in cells immediately following exposure to glycerol (Figure 6 and 7). In addition, ultrathin sections of the cytoplasm of glycerol-treated cells show vesicle-like bodies in membrane-bound inclusions and numerous vacuoles which closely resemble those reported by Rahman & Wright (1975) in mouse hepatocytes and Kupffer cells after in vivo injection of PC–cholesterol vesicles. Similar effects of glycerol on membranes have been observed previously in leukocytes, which exhibit increased ruffling, filopodia formation, and membrane internalization upon exposure to glycerol (Norberg, 1970).

The results of the experiments with metabolic inhibitors (Table II) indicate that glycerol stimulation of liposome delivery occurs via an energy-dependent pathway which is largely unaffected by agents that disrupt microtubule structure. The results mimic those obtained for sucrose uptake in AGMK cells and are consistent with generally accepted view on the inhibitor sensitivity of endocytosis in mammalian cells (Silverstein et al., 1977, 1978). However, on the basis of inhibitor effects observed, we cannot exclude the involvement of other metabolically dependent cellular processes which may be responsible for the intracellular uptake of liposome contents. While both inhibitor and morphological studies suggest that glycerol stimulates the bulk of liposome uptake via endocytosis, it is still possible that some alternate pathway such as membrane fusion could be responsible for the infection of cells by encapsulated SV40 DNA. It is also quite possible that the mechanism of uptake may vary with different types of polymers, liposomes, or cells. In this respect, it should be noted that Szoka et al. (1981) have recently suggested that poly(ethylene glycol) (PEG) treatment stimulates the cellular uptake of glycolipid-containing liposomes (in the presence of lectins) via their fusion with the plasma membrane.

The effect of glycerol is not limited to AGMK cells; the liposome-mediated delivery to mouse L cells or to HeLa cells of SV40 or HSV-TK DNA (monitored by T-antigen production or the appearance of colonies in HAT media, respectively) is also increased by exposure to glycerol (unpublished observations). In addition, glycerol treatment shows a similar effect on the expression of entrapped RNA; liposome-encapsulated polio RNA shows the same proportional enhancement by glycerol following incubation with either AGMK or HeLa cells (data not shown). Finally, it should be emphasized that the glycerol treatment is not limited to enhancing delivery of liposome-encapsulated nucleic acids,

since the intracellular delivery of the fluorescent dye, carboxyfluorescein, entrapped in PS vesicles, is enhanced greatly by glycerol treatment (Figure 8). This method should prove generally useful in increasing the delivery of liposome-encapsulated molecules (drugs, enzymes, etc.) to different cell types.

The use of CF delivery as an assay for monitoring liposome uptake by cells has been controversial due to problems associated with the uptake of the free dye by cells (Szoka et al., 1979). However, the strong correlation observed between CF and SV40 DNA delivery to AGMK cells with respect to liposome composition, glycerol treatment, and the effect of metabolic inhibitors indicates that under the specific incubation conditions described here, CF delivery can be used to study liposome–cell interactions. It should be noted that in the absence of glycerol treatment we could not detect appreciable intracellular fluorescence, indicating this method is far less sensitive than the infectivity assays.

The observation that chloroquine, a drug known to alter lysosomal pH and activity (De Duve et al., 1974), enhances infectivity of liposome-entrapped SV40 DNA (approximately 4–6-fold) following glycerol treatment suggests the involvement of a lysosomal processing step in glycerol-treated cells. It also provides additional evidence that at least some of the glycerol-stimulated cellular uptake of liposome-encapsulated SV40 DNA occurs via an endocytotic pathway. We cannot exclude additional effects of chloroquine on membrane structure and function, though a nonspecific effect of chloroquine on DNA replication (Cohen & Yielding, 1965) is unlikely; chloroquine also stimulates the infectivity of liposome-encapsulated polio RNA following glycerol exposure, and NH_4Cl, which also raises lysosomal pH but does not bind to DNA, also increases liposome-encapsulated SV40 DNA infectivity (data not shown). In view of a number of investigations which indicate that chloroquine can inhibit the lysosomal degradation of proteins (Wibo & Poole, 1974; Carpenter & Cohen, 1976; Ascoli, 1978; Goldstein et al., 1979) and uncoating of certain viruses (Helenius et al., 1980; Norkin & Einck, 1978), it seems likely that chloroquine increases SV40 DNA infectivity by preventing its degradation in lysosomes. The protective effect of chloroquine could be exerted directly as a consequence of raising lysosomal pH or disabling specific degradative enzymes, or indirectly by preventing the fusion of endocytotic vesicles with lysosomes. In either case, there would be an enhanced possibility of escape of the liposome-encapsulated SV40 DNA into the cytoplasm (and eventually the nucleus), either by fusion of the liposome directly with the phagolysosomal membrane or by some nonspecific breakdown of these membranes.

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Sequence and Structure in Double-Stranded Ribonucleic Acid: $(A\text{-}G\text{-}C\text{-}U)_2$ and $(A\text{-}C\text{-}G\text{-}U)_2$†

Edward Bubienko, Mary Alice Uniack,‡ and Philip N. Borer*

ABSTRACT: Comparative studies of the thermally induced helix–coil transition in ribosyl $(A\text{-}G\text{-}C\text{-}U)_2$ and $(A\text{-}C\text{-}G\text{-}U)_2$ are described. Ordered structures form at low temperatures where the ribofuranose rings adopt the 3′-endo conformation and both oligomer helices have base-paired stacking arrangements qualitatively similar to the A-RNA family configuration. Especially for $(A\text{-}C\text{-}G\text{-}U)_2$, there is a lack of quantitative agreement between the A-family base overlap and the ^1H NMR data; ring-current and atomic diamagnetic anisotropies using A-form structures fail to predict five of the seven aromatic C–H resonances within 0.2 ppm. The NMR results are in better agreement with the A form for $(A\text{-}G\text{-}C\text{-}U)_2$. For both oligomers, the changes in chemical shift for the anomeric (H1′) resonances indicate substantial (≥20°) changes in the average glycosidic torsion angle upon base pairing and stacking for the adenosine and cytidine residues; this angle in uridine and guanosine residues must change only slightly.

Oligonucleotides as models for double-stranded RNA have been studied extensively since the pioneering work of Thach (1966), Martin et al. (1971), and Uhlenbeck et al. (1971), who developed procedures for the preparation of oligomers having a defined sequence. Kearns (1977) and Kallenbach & Berman (1977) have reviewed much of the subsequent literature that developed in the application of physical methods to determining structural and dynamic features of these defined model systems.

NMR[1] is one of the few solution methods that can provide structural information on a detailed atom-by-atom level. Until now much of its potential has gone unrealized because of problems in theoretical interpretation of chemical shift changes when the oligomers undergo a helix–coil transition. It has been difficult to connect these large (up to 1.5 ppm) changes to geometrical features in anything but a qualitative fashion. Nevertheless, it has been shown that these oligo-RNA duplexes conform to the standard ^3E (3′-endo) furanose ring pucker and the general features of the A-RNA family of structures determined by X-ray diffraction in the solid state. Much of the difficulty in the quantitative interpretation of the NMR data stems from an inadequate theoretical description of the local magnetic anisotropy of such functionalities as the phosphate and the furanose ring oxygen as well as effects of solvent exclusion, hydrogen bonding, multiple aggregation, etc. described by Borer et al. (1975).

In several important papers, Giessner-Prettre, Pullman, and co-workers (1976, 1977a,b), Arter & Schmidt (1976), and Kan et al. (1979) accurately considered the spatial dependence of ring current and atomic diamagnetic anisotropies on the aromatic ring and furanose protons. It occurred to us that these new tools in a careful comparative approach could allow us to probe the structural details of oligomer helices as never before. The simple tetranucleotide duplexes $(A\text{-}G\text{-}C\text{-}U)_2$ and $(A\text{-}C\text{-}G\text{-}U)_2$ are easily synthesized in good yield, have simple, easily assigned NMR spectra because of their self-complementary nature, and, in the comparison, might have nearly identical characteristics with respect to the phosphate and furanose anisotropies, solvent exclusion, hydrogen bonding, multiple aggregation, etc. that plague studies that depend on de novo application of NMR principles to a single oligomer.

We find that both duplexes belong to the A-RNA family of structures with a ^3E sugar pucker and the general features of A-form base stacking. However, $(A\text{-}C\text{-}G\text{-}U)_2$ clearly does

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[1] Abbreviations and symbols used: NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; FT, Fourier transform; δ, chemical shift; $J_{1'-2'}$, ribose H1′–H2′ coupling constant; J_{5-6}, base H5–H6 coupling constant; Pu, purine; Py, pyrimidine; A-G-C-U and A-C-G-U, ribosyl ApGpCpU and ApCpGpU, respectively (the standard 3′–5′ phosphodiester linkage is implied in this notation for these and other oligoribonucleotides); CDP, cytidine 5′-diphosphate; GDP, guanosine 5′-diphosphate; UDP, uridine 5′-diphosphate; PNPase, primer-independent polynucleotide phosphorylase (*M. luteus*, EC 2.7.7.8); PNPase P, primer-dependent polynucleotide phosphorylase; RNase A, bovine pancreatic ribonuclease (EC 3.1.27.5); RNase T_1, ribonuclease T_1 (*A. oryzae*, EC 3.1.27.5); BAPase, bacterial alkaline phosphatase (*E. coli*, EC 3.1.3.1); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEAB, triethylammonium bicarbonate; EDTA, ethylenediaminetetraacetic acid; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; DEAE-Sephadex, diethylaminoethyl-Sephadex A-25.

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