

# INTERACTION OF PHOSPHOLIPID VESICLES WITH CULTURED MAMMALIAN CELLS

## I. Characteristics of Uptake

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### ABSTRACT

The interaction of monolayer cultures of Chinese hamster V79 cells with artificially generated, unilamellar lipid vesicles (~500 Å diameter) was examined. Vesicles prepared from a variety of natural and synthetic radiolabeled phosphatidyl cholines (lecithins) were incubated with V79 cells bathed in a simple balanced salt solution. After incubation, the cells were analyzed for exogenous lipid incorporation. Large quantities (~10<sup>9</sup> molecules/cell/h) of lecithin became cell associated without affecting cell viability. The effects of pH, charged lipids, and the influence of the vesicle lipid phase transition on the uptake process were examined. Glutaraldehyde fixation of cells before vesicle treatment, or incubation in the presence of metabolic inhibitors, failed to reduce the lecithin uptake by more than 25–50%, suggesting that the lipid uptake is largely energy independent.

Cells in sparse culture took up about ten times more lipid than dense cultures. Prolonged incubation (> 15 h) of sparse cell cultures with lecithin vesicles resulted in significant cell death, while no deleterious effect was found in dense cultures, or with 1:1 lecithin/cholesterol vesicles.

When vesicle-treated cells were homogenized and fractionated, about 20–30% of the exogenous lipid was found in the plasma membrane fraction, with the remainder being distributed into intracellular fractions. Electron microscope radioautography further demonstrated that most of the internalized lipid was present in the cytoplasm, with little in the nucleus.

These results are discussed in terms of possible modification of cell behavior by lipid vesicle treatment.

In order to delineate some of the relationships between membrane composition and the functional states of mammalian cells, we are exploring the possibility of introducing into the plasma membrane of a living, intact cell, macromolecules derived from the plasma membrane of a second cell type. Since the alteration of the cell surface may have a profound influence on the physiology

of the cell and possibly its state of differentiation, the significance of the proposed studies is readily apparent. For example, such well-described membrane functions as hormone sensitivity or electrical excitability might be induced in cells lacking them by transfer of the appropriate receptors onto the recipient cells. Since most membrane macromolecules are hydrophobic and therefore readily

soluble only within the two-dimensional confines of the lipid membrane with which they are normally associated, one approach to the membrane modification referred to above would be through the use of artificially generated lipid vesicles, into which the appropriate macromolecules had been reconstituted. Such vesicles could then be used in performing the transfer of the foreign membrane component to the recipient cell by some process such as vesicle-cell fusion. The use of lipid vesicles (liposomes or phospholipid dispersions) for this purpose is particularly attractive because the system is well-defined, chemically and physically, and readily adaptable to membrane reconstitution studies (6).

As a first step toward the alterations in surface properties described above, we have examined the characteristics of the interaction of chemically and physically well-defined lipid vesicles, containing no reconstituted proteins, with cultured mammalian cells, bathed in a simple balanced salt solution. Detailed studies of the molecular mechanism of this interaction are presented in the second, accompanying paper in this series (13).

## MATERIALS AND METHODS

### *Cell Culture*

The Chinese hamster V79 lung cell line, derived by Ford and Yerganian (5), was used in this study. Cells were grown on Falcon plastic tissue culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in Eagle's Minimal Essential Medium supplemented with 2% lactose and 15% fetal calf serum at 37°C in an atmosphere of 5% CO<sub>2</sub> in air (26). Cells were counted in a Fuchs-Rosenthal hemocytometer chamber.

### *Lipids and Lipid Vesicles*

Egg yolk lecithin (EYL) was isolated from chicken egg yolks (15) and purified by chromatography on silicic acid (19). The synthetic phosphatidyl cholines, dioleoyl lecithin (DOL) and dimyristoyl lecithin (DML), were prepared by hydrolysis of EYL to L- $\alpha$ -glyceryl phosphoryl choline and free fatty acids (2), followed by reacylation using the appropriate fatty acid anhydride (18). Fatty acid anhydrides were prepared from myristic or oleic acids (Applied Science Labs, Inc., State College, Pa.) as described elsewhere (22). The lecithins obtained from these procedures showed a single component on thin-layer chromatography (TLC) (23).

Phosphatidyl glycerol was synthesized from EYL using phospholipase D, and purified as described elsewhere (16). Phosphatidyl serine (Supelco, Inc., Bel-

fonte, Pa.), stearyl amine (Koch Light Laboratories, Buckinghamshire, England) and cholesterol (Applied Science Labs, Inc.) were purchased from commercial sources and used without further purification.

Dioleoyl phosphatidyl [<sup>14</sup>C]choline was a generous gift from Dr. E. A. Dawidowicz, Harvard Medical School, Boston, Mass.

Di[9,10-<sup>3</sup>H]oleoyl lecithin and di[<sup>14</sup>C]myristoyl lecithin were prepared as described above, using the appropriate labeled fatty acid (New England Nuclear, Boston, Mass.) as a starting material. EYL was labeled by adding a trace amount of ~ 5 Ci/mmol [<sup>3</sup>H]distearoyl lecithin (DSL) to EYL to give a product with a final specific activity of 1 mCi/mmol. [<sup>3</sup>H]DSL was prepared by catalytic hydrogenation of DOL by the Tritium Labeling Laboratory, New England Nuclear, and subsequently purified in our laboratory by silicic acid chromatography. [4-<sup>14</sup>C]cholesterol was purchased from New England Nuclear.

All lipids were stored under nitrogen in sealed ampoules at -15°C, and periodically examined for purity by TLC.

Lipid vesicles were prepared as follows. Radiolabeled lecithin (100 mg) of known specific activity was suspended in approximately 7 ml of Gey's balanced salt solution (hereafter Gey's). The suspension was then ultrasonically irradiated with a 20-kc Branson Model W-185 Sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at power level 4 in a jacketed vessel maintained at constant temperature by a circulating water bath. All sonications were carried out in an argon atmosphere. Sonication times and temperatures were as follows: EYL (2 h, 2°C); DOL (1 h, 2°C); DML (30 min, 41°C). After sonication, the lipid dispersion was centrifuged (10,000 × g, 10 min) and then separated (7) into multilamellar and unilamellar fractions by gel filtration on a 2.5 × 40 cm Sepharose 4B column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The unilamellar vesicle fractions were pooled and diluted to a final concentration of 1 mg phospholipid/ml in Gey's salt solution.

In the case of vesicles comprised of more than one lipid species, the lipids were first dissolved in an organic solvent to assure intimate mixing of the components. The solvent was then evaporated under N<sub>2</sub>, and the vesicles were prepared as described above.

### *Vesicle Uptake Experiments*

In a typical experiment, a tissue culture plate containing a monolayer of approximately 10<sup>7</sup> cells was first washed extensively with Gey's to remove all culture medium from the dish. Except as noted, cells were near confluence (~ 4 × 10<sup>6</sup> cells/cm<sup>2</sup>). Next, the radiolabeled vesicle suspension, also prepared in Gey's, was added to the culture dish, and the cells were incubated with the vesicle suspension either at 2°C or 37°C for different periods of time. After the incubation period, the vesicle suspension was aspirated from the culture dish, and the

plates were washed three times with Gey's. The cells were then removed from the tissue culture plates by brief trypsinization. The resulting cell suspension was centrifuged and resuspended in fresh balanced salt solution for a total of three washes. Before the last centrifugation, a cell count was made. The final cell pellet was solubilized with NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) and the total radioactivity of the cell pellet determined using liquid scintillation counting. From the activity of the sample, the specific activity of the phospholipid used, and the cell number, the total number of phospholipid molecules taken up per cell was calculated. Reproducibility was  $\pm 5\%$ .

Experiments on the effect of pH on lipid uptake from vesicles were done in Gey's salt solution containing 10 mM concentrations of the following buffers (Calbiochem, San Diego, Calif.): 2-(*N*-morpholino)-ethanesulfonic acid (pK 6.15); morpholinopropanesulfonic acid (pK 7.2); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pK 7.55); and *N*-tris-(hydroxymethyl)-methylglycine (pK 8.15). Solutions were adjusted to the required pH by titration with concentrated NaOH or HCl.

### Membrane Isolation and Analysis

The plasma membranes from cells treated 1 h at 37°C with radiolabeled lipid vesicles were isolated according to the technique of Wallach and Kamat (29). Cells were equilibrated in a cell disruption bomb (Artisan Industries Inc., Waltham, Mass.) at 0–4°C in a N<sub>2</sub> atmosphere (1,000 psi) for 20 min before disruption by dropwise release from the pressure homogenizer. The crude homogenate was fractionated, as described elsewhere (29), into plasma membrane vesicles and various intracellular fractions. The isolated membranes were judged to be more than 90% pure by electron microscopy, and the presence of the plasma membrane marker, 5'-nucleotidase. No cytochrome oxidase, NADH diaphorase, or lactate dehydrogenase activities were found in the purified plasma membrane fraction. The isolated membranes were lyophilized and their lipids extracted with chloroform-methanol (2/1, vol/vol) after the method of Folch et al. (4). The amount of lipid extracted was quantified by the method of Saito and Sato (20) and the radioactivity determined by liquid scintillation counting. From the specific activity of the exogenously supplied radiolabeled lipid, the percentage of the extracted lipids which were radiolabeled was calculated. Similar lipid analyses were carried out on intact, vesicle-treated cells. TLC of the extracted lipids was carried out with precoated silica gel plates using chloroform:methanol:acetic acid:H<sub>2</sub>O, 25:5:4:2 (vol/vol) as the developing solvent. Spots were visualized with I<sub>2</sub> vapor and scraped from the thin-layer plate for radioactivity analysis.

### Electron Microscopy

All electron micrographs were obtained with a Hitachi HU-11E-1 electron microscope operating at 75 kV.

Cultures of vesicle-treated cells were fixed at 4°C for 1 h to 12 h in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.4, rinsed in buffer, postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer, and rinsed in distilled water. The cells were then stained for 12–24 h in 0.5% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Epon. To minimize heat-induced buckling of the Falcon dishes, polymerization was initiated at 40°C for 24 h and the plates were then transferred to 57°C for 24–48 h. The petri dishes were then split away from the embedding plastic, which was further polymerized at 70°C for 24 h. Serial sections were obtained with a Porter-Blum MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newton, Conn.), and mounted on Formvar-coated grids (200 mesh). Sections were stained with lead citrate before examination in the electron microscope.

For electron microscope autoradiography, unilamellar vesicles were prepared from a mixture of DOL containing a trace amount of radiolabeled [<sup>3</sup>H]DSL to give a product with a final specific activity of ~40 mCi/mmol. This material was purified by silicic acid chromatography immediately before preparation of the lipid vesicles, and the latter were used for the treatment of cells within 24 h of their preparation. Electron microscope autoradiographs were prepared following the procedure of Caro et al. (3). Ilford L-4 (Ilford Ltd., Essex, England) emulsion, diluted 1:2 with distilled water, was applied to prepared grids containing sectioned material, using the "loop" method. Test grids were examined periodically during application of the emulsion to verify that each grid was coated reproducibly with a uniform monolayer of silver halide crystals. Specimens were stored desiccated at 4°C in an argon atmosphere for 1–2 mo, before photographic development with Kodak Microdol-X (Eastman Kodak, Co., Rochester, N. Y.).

### RESULTS

The average diameter of the vesicles used in the present studies was estimated to be about 500 Å by electron microscope techniques (11). These studies further revealed that the population of vesicles was homogeneous in size and totally unilamellar (that is, the aqueous interior of each vesicle was bounded by a single lipid bilayer). No degradation of the lipids following sonication could be detected by TLC, and vesicle suspensions prepared by an alternate technique (1) which obviates the sonication step gave results identical to those reported in this paper.

Typical uptake data obtained for V79 cells incubated with preparations of unilamellar vesicles generated from DOL and DML vesicles are shown in Fig. 1. Approximately 10<sup>8</sup> molecules of phospholipid per cell became cell associated in a relatively short period. This observation is consist-

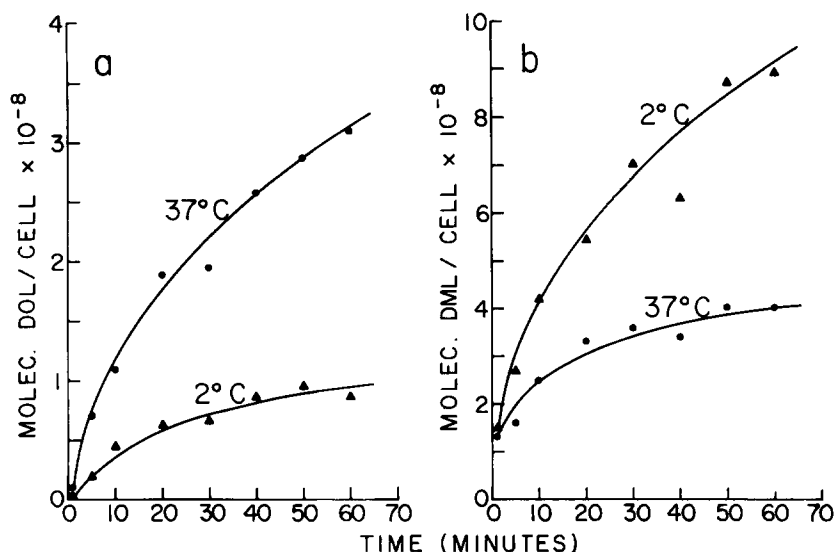


FIGURE 1 Uptake of (a) [ $^3\text{H}$ ]DOL and (b) [ $^{14}\text{C}$ ]DML by Chinese hamster V79 cells from unilamellar lipid vesicles at  $2^\circ\text{C}$  ( $\blacktriangle$ — $\blacktriangle$ ) and  $37^\circ\text{C}$  ( $\bullet$ — $\bullet$ ). Activities were  $1.64 \times 10^{-13}$  cpm/molecule for DOL and  $2.24 \times 10^{-13}$  cpm/molecule for DML. Cells were near confluence ( $\sim 4 \times 10^6$  cells/cm $^2$ ) at time of the experiment.

ent with our earlier report on radiolabeled EYL vesicles (14) which are chemically less well defined than DOL or DML. In order to examine whether the lipid uptake represents the uptake of intact lecithin molecules, cells were treated with vesicles generated from a mixture of di[9,10- $^3\text{H}$ ]oleyl lecithin and dioleoyl phosphatidyl [ $^{14}\text{C}$ ]choline. The uptake of  $^{14}\text{C}$  and tritium by vesicle-treated cells was measured after a 1-h treatment at  $37^\circ\text{C}$ , and is given in Table I. The ratio of [ $^{14}\text{C}$ ]choline which became cell associated is nearly identical to the  $^3\text{H}$  to  $^{14}\text{C}$  ratio in the applied vesicle suspension, suggesting the incorporation of intact lecithin molecules.

The amount of lecithin taken up per cell depended on the concentration of vesicles applied to the cells. Thus, for example, cells incubated with vesicles applied at a concentration of 1 mM lipid phosphorus took up about an order of magnitude more phospholipid than cells incubated with vesicles at a concentration of 0.1 mM lipid. Under the conditions of the experiments reported in this paper, the lipid vesicles were always applied in great excess with respect to the number of cells present ( $\sim 10^8$  vesicles/cell), and the uptake, therefore, represents only a small fraction, typically  $\sim 0.1\%$ , of the total amount of lipid incubated with the cells. It should be noted, however, that this number is an arbitrary one since increasing the

TABLE I  
Uptake of Lecithin at  $37^\circ\text{C}$  by Chinese Hamster V79 Cells Treated with Doubly-Labeled Lecithin Vesicles\*

[ $^{14}\text{C}$ ]choline moiety found in treated cells, cpm	1,648
[ $^3\text{H}$ ]oleic acid moiety found in treated cells, cpm	1,986
$^3\text{H}$ cpm/ $^{14}\text{C}$ cpm in treated cells	1.21
$^3\text{H}$ cpm/ $^{14}\text{C}$ cpm in applied vesicles	1.18

\* A tissue culture plate containing  $\sim 10^7$  cells was treated for 1 h at  $37^\circ\text{C}$  with a vesicle suspension (1 mg total phospholipid/ml) prepared from a mixture of dioleoyl phosphatidyl[ $^{14}\text{C}$ ]choline and di[9,10- $^3\text{H}$ ]oleoyl phosphatidyl choline.

volume of vesicle suspension added to the monolayer of cultured cells, while maintaining the vesicle concentration constant, has no effect on the amount of lipid uptake.

The uptake of exogenous phospholipid from vesicles by V79 cells was independent of pH over the interval 6.8–8.7. Furthermore, the introduction of charge into the vesicles by cosonication of lecithin with charged lipids (e.g., 10% phosphatidyl serine; 10% phosphatidyl glycerol [both negatively charged]; or 10% stearyl amine [positively

charged]) altered neither the kinetics nor the absolute amounts of lecithin uptake by the cells.

In order to examine the influence of lipid fluidity on the uptake process, the incorporation of phospholipid into V79 cells from vesicles prepared from DOL and DML has been compared. This phospholipid pair was chosen because the gel-liquid crystalline phase transition temperatures ( $T_c$ ) for DOL ( $-20^\circ\text{C}$ ) and DML ( $+25^\circ\text{C}$ ) are very different. Thus vesicles prepared from DOL are in a fluid, liquid-crystalline state at both  $2^\circ\text{C}$  and  $37^\circ\text{C}$ , whereas DML vesicles are in a fluid state at  $37^\circ\text{C}$  and a relatively solid or gel phase at  $2^\circ\text{C}$ . The results of two different uptake experiments with these different vesicle preparations are shown in Fig. 1. At  $37^\circ\text{C}$ , the absolute amounts of lecithin uptake by vesicle-treated cells are nearly equal for DOL or DML vesicles. However, phospholipid from DOL vesicles was more effectively taken up by cells at  $37^\circ\text{C}$  than at  $2^\circ\text{C}$ , while the opposite was true for DML vesicles; about two-fold more DML was incorporated into cells at  $2^\circ\text{C}$  than at  $37^\circ\text{C}$ . This effect is even more pronounced if it is considered that the normal reduction of lipid uptake from vesicles over this temperature interval in the absence of a phase transition is about 50%.

The influence of cell density on the uptake of lipid from phospholipid vesicles is shown in Fig. 2. In this experiment, DOL vesicles were incubated (1 h at  $25^\circ\text{C}$ ) with cell cultures seeded at different initial densities. The abscissa of Fig. 2 represents the cell density on the tissue culture plate at the time that incubation with vesicles was carried out. It is seen that cells at very low densities incorporate about an order of magnitude more phospholipid than cells near confluence ( $22.5 \times 10^8$  molecules DOL/cell at  $\sim 10^8$  cells/cm $^2$  vs.  $2.2 \times 10^8$  molecules DOL/cell at  $\sim 4 \times 10^8$  cells/cm $^2$ ).

Prolonged incubation of cells with vesicles produced from EYL or DOL can have a profound effect on their ability to exclude vital stains. Fig. 3 shows the percent trypan blue-staining cells as a function of the incubation time with lecithin vesicles. No effect on the ability to exclude dye was observed for incubation periods up to 6 h, and normal cell growth resumed upon replacement of the vesicle suspension with fresh culture medium. However, prolonged incubation resulted in 100% stained cells after a 15-h incubation with sparse cultures, 95% stained cells after 24-h incubation with medium density cultures, while confluent cultures showed no difference in their ability to exclude dye from control cultures incubated in a

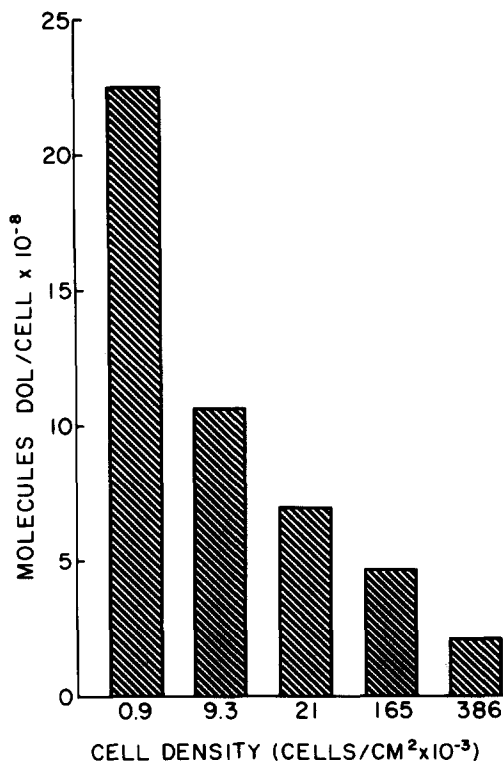


FIGURE 2 Influence of cell density on the uptake of DOL from unilamellar vesicles by Chinese hamster V79 cells. 1-h incubation at  $25^\circ\text{C}$ ; cell density was determined at the time that the uptake experiment was carried out.

balanced salt solution alone. Cultures containing nearly 100% stained cells also contained large numbers of floating cells as well as attached cells which were judged unhealthy because of their very rounded and ruffled appearance. Furthermore, replicate plates, not treated with dye, but to which fresh culture medium was added after incubation with vesicles, did not grow, nor did they exclude dye 24 h later. No differences in cloning efficiencies could be observed for vesicle-treated vs. untreated control cells in high density cultures.

Cells treated with 1:1 lecithin/cholesterol vesicles were indistinguishable from control, untreated cell cultures, and showed only about 5% stained cells after 15 h of incubation, regardless of cell density. Uptake experiments with vesicles comprised of a 1:1 mixture of radiolabeled lecithin and cholesterol showed that both components became cell associated in amounts comparable to the uptake observed from vesicles comprised of lecithin alone (13).

After a 1-h incubation period at  $37^\circ\text{C}$  with

vesicles comprised of di[9,10-<sup>3</sup>H]oleyl lecithin, all the radioactivity taken up by the cells remained chloroform-methanol extractable, and was predominantly (~90%) lecithin. About 20–30% of this radioactivity appeared in the plasma membrane fraction, while the remainder was distributed into various crude intracellular fractions as follows: ~10% in the endoplasmic reticulum, ~10–20% in the pellet of a low speed centrifugation (20,000 g; 15 min) containing nuclei, mitochondria, and lysosomes, and ~50% in the cytosol or supernatant fraction of a high speed centrifugation (100,000 g; 45 min). Analysis of the lipids from the plasma membrane and endoplasmic reticulum fractions by TLC showed that the foreign phospholipid incorporated into the plasma membrane was not degraded but remained as lecithin, while in the endoplasmic reticulum ~50% of the radioactivity appeared as free fatty acid. Lipid analysis showed that approximately 2% of the total cell lipids were radioactive following a 1-h vesicle treatment. A similar analysis of the lipid fraction from isolated

plasma membranes showed that the radioactive lipids represented about 6% of the total membrane lipids, indicating some enrichment of the exogenous lipid in the plasma membrane fraction of the cell.

The influence of various inhibitors on the uptake of lecithin from vesicles by V79 cells is summarized in Table II. When cells were incubated with vesicles in Gey's salt solution, or in Gey's containing 30 mM NaN<sub>3</sub> or 0.5 mM 2,4-dinitrophenol (DNP), the amounts of lecithin taken up per cell were, within experimental error, indistinguishable. This observation holds true both at 2°C and at 37°C. If, however, the carbon source, dextrose, was omitted from the balanced salt solution and the cells were preincubated in this "modified Gey's" solution for 2 h before the addition of lipid vesicles, there was a substantial reduction in the amount of lecithin uptake at 37°C ( $3.9 \times 10^8$  molecules/cell) compared to control cells incubated in Gey's alone ( $5.1 \times 10^8$  molecules/cell). Cells pretreated in modified Gey's containing either NaN<sub>3</sub> (30 mM) or DNP (0.5 mM) showed a further slight reduc-

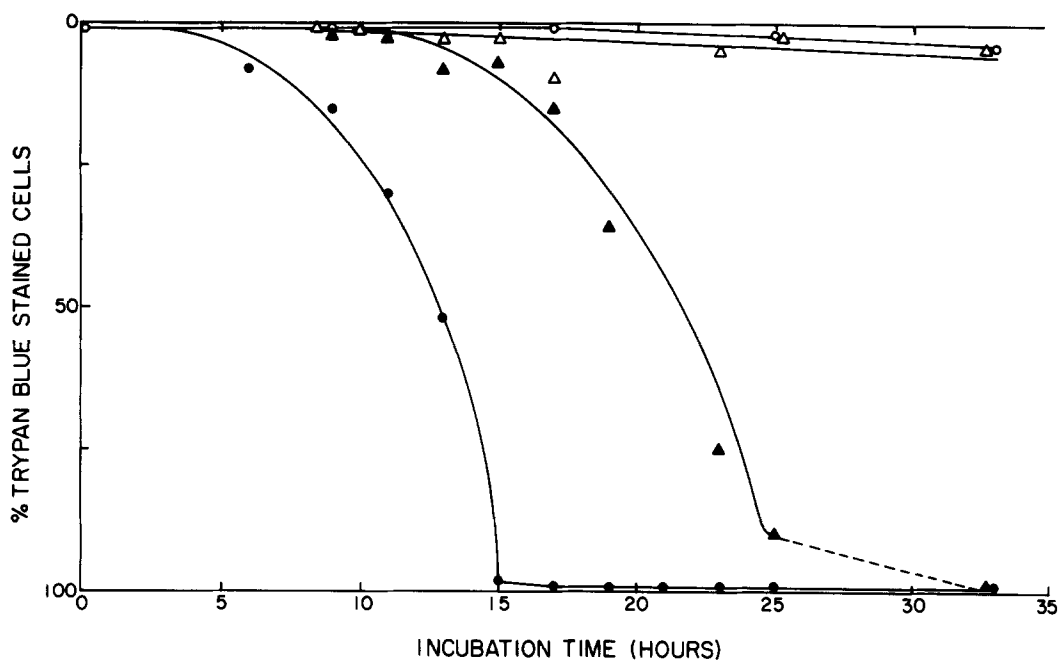


FIGURE 3 Effect of prolonged incubation (37°C) of V79 cells with DOL or EYL lipid vesicles (1 mg phospholipid/ml) on the trypan blue staining of vesicle-treated cells. Vesicles were incubated with cells at low ( $\sim 10^8$  cells/cm<sup>2</sup>; ●—●), medium ( $\sim 2 \times 10^4$  cells/cm<sup>2</sup>; ▲—▲) and high ( $\sim 4 \times 10^6$  cells/cm<sup>2</sup>; △—△) density. Control plates, using all three cell densities (O—O), were incubated in balanced salt solution alone containing no lipid vesicles. Cells treated with 1:1 EYL: Cholesterol vesicles gave results identical to those of control, untreated cultures.

tion in their ability to take up exogenous phospholipid upon incubation with lipid vesicles at 37°C. The pretreatments described above had no effect on the lipid uptake at 2°C, and all values given in Table II at 2°C are, within experimental error, identical.

Cells pretreated with 2% glutaraldehyde (2 h at 4°C) before uptake showed about a 25–50% reduction in the amount of phospholipid taken up after a 1-h incubation (at 2°C or 37°C) period. These results strongly suggest that a substantial fraction of the uptake is a physical process, and not one dependent on metabolic energy.

Electron microscope autoradiographs of V79 cells treated with DOL vesicles containing trace amounts of [<sup>3</sup>H]DSL are given in Fig. 4. A separate background grid containing no sectioned material was processed simultaneously with each specimen grid. The background was random and found to be less than 5 grains per grid square. Cells treated at 37°C with lipid vesicles for 5 min or 1 h showed qualitatively similar distributions of radiolabeled lecithin, namely, most of the label appeared to be internalized in the cytoplasm of the cell, with little if any radioactivity in the nucleus and relatively small amounts associated with the surface membrane (Fig. 4 a). A possible accumulation of label over cytoplasmic lipid droplets was also seen (Fig. 4 b).

## DISCUSSION

While this study deals only with the interaction of phospholipid vesicles with Chinese hamster V79 cells, the uptake data obtained are representative

of our observations with other established cell lines grown on tissue culture plates (e.g., Chinese hamster ovary; mouse L929; BALB/c3T3) and primary cells in suspension (e.g., spleen; bone marrow; macrophage; T cells; thymus and lymphoblast cells from CBA mice). In all cases, a very substantial quantity of phospholipid from the applied vesicle suspension is taken up by vesicle-treated cells. From the uptake data in Fig. 1, and from estimates of the number of lipid molecules per vesicle (7), it is calculated that enough lipid to account for roughly 10<sup>5</sup>–10<sup>6</sup> vesicles/cell has become cell associated after a 1-h incubation period.

It is important to note that, in most of the experiments described in this and the accompanying paper (13), the lecithins used were radiolabeled in the fatty acid moiety. Thus, it is possible that hydrolysis of the exogenously supplied lecithin occurs at the cell surface, and that the uptake phenomena observed in these studies represent the uptake of free fatty acid which is later resynthesized into lecithin and other lipids. In order to test this possibility, vesicles comprised of a mixture of two species of DOL, one labeled with <sup>14</sup>C in the choline moiety and another with [<sup>3</sup>H]oleic acid in the fatty acid moiety of the molecule, were prepared and used in uptake experiments. The data, presented in Table I, show that, at 37°C, both labels became cell associated in amounts equivalent to their relative proportions in the applied vesicle suspension. This strongly suggests that intact lecithin molecules are taken up by vesicle-treated cells.

Phospholipid will swell spontaneously in an

TABLE II  
*Effect of Metabolic Inhibitors on Phospholipid Uptake by Chinese Hamster V79 Cells Treated with Lipid Vesicles*

Pretreatment*	Incubation medium†	Molecules DOL/cell × 10 <sup>-8</sup> after 1-h incubation	
		2°C	37°C
—	Gey's	1.6 ± 0.1§	5.2 ± 0.3
—	Gey's + 30 mM NaN <sub>3</sub>	1.6 ± 0.1	4.7 ± 0.3
—	Gey's + 0.5 mM DNP	1.4 ± 0.1	4.9 ± 0.3
Gey's	Gey's	1.4 ± 0.1	5.1 ± 0.3
Gey's - dextrose	Gey's - dextrose	1.5 ± 0.1	3.9 ± 0.2
Gey's - dextrose + 30 mM NaN <sub>3</sub>	Gey's - dextrose + 30 mM NaN <sub>3</sub>	1.5 ± 0.1	3.3 ± 0.2
Gey's - dextrose + 0.5 mM DNP	Gey's - dextrose + 0.5 mM DNP	1.6 ± 0.1	3.6 ± 0.2

\* Pretreatment was carried out for 2 h at 2° or 37°C, depending on temperature of subsequent incubation with lipid vesicles.

† Incubation was carried out for 1 h with vesicles prepared from DOL.

§ Mean Value ± SD

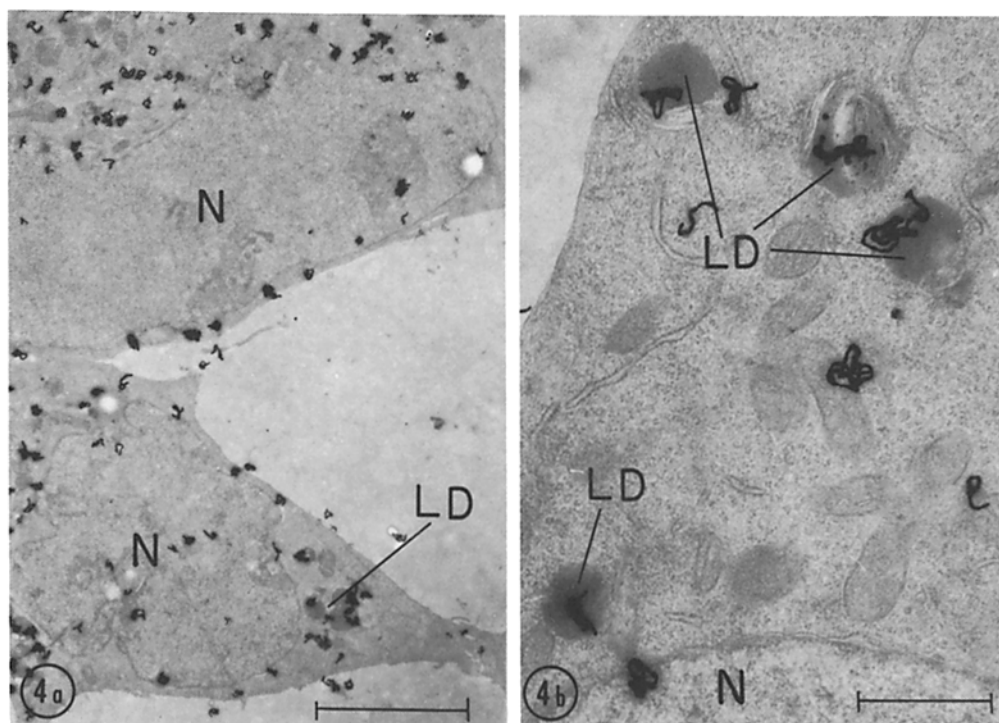


FIGURE 4 Electron microscope autoradiographs of V79 cells treated with [ $^3\text{H}$ ]lecithin vesicles at  $37^\circ\text{C}$  for 1 h. *N* = nucleus; *LD* = lipid droplets, (a)  $\times 4,000$ . Bar is  $5\ \mu\text{m}$ . (b)  $\times 17,000$ . Bar is  $1\ \mu\text{m}$ .

aqueous environment to form liquid crystals when the temperature exceeds the melting temperature of the hydrocarbon chains within the structure. This gel-liquid crystalline phase transition can be induced by raising the temperature, decreasing the chain length of the hydrocarbon moiety, increasing the extent of unsaturation, or introducing substituents which promote disorder in the hydrocarbon region (9, 10). No significant differences in lipid uptake from vesicles comprised of various lecithins were found when the experiment was carried out above the phase transition temperature of the vesicle lipid. Thus, the absolute amounts of lecithin taken up per cell after vesicle treatment were very similar for uptake studies carried out at  $37^\circ\text{C}$  with EYL, DOL, or DML vesicles. In contrast, the behavior of cells treated with DOL ( $T_c = -20^\circ\text{C}$ ) or DML ( $T_c = +25^\circ\text{C}$ ) vesicles at  $2^\circ\text{C}$  shows that the uptake process is greatly enhanced below the phase transition of the exogenous lipid (Fig. 1 *b*). A similar phenomenon has been observed by other laboratories studying the uptake of free fatty acids by a variety of mammalian cells (24, 25). Although no data on the temperature dependence of this

process are available, the effects of fatty acid chain length and degree of unsaturation have been examined. It was shown that the uptake of saturated fatty acids increases with increasing chain length, and that saturated fatty acids of a given chain length are taken up to a greater extent than the corresponding unsaturated fatty acids. These results have been interpreted as reflecting the differences in binding of free fatty acid to the target membrane. On the basis of this work, it is reasonable to assume that a similar relation between the fluidity of the lipid vesicle and its ability to bind to the surface membrane of the cell may also hold. Such a binding must be the first step in any proposed mechanism involving an interaction between the cultured cell and lipid vesicle, as discussed in the subsequent paper (13).

The uptake of phospholipid from lipid vesicles by sparse cell cultures was about an order of magnitude higher than that exhibited by confluent ones. Several possible explanations for this marked cell-density dependent phenomenon are considered. (a) Scanning electron microscopic examination of the surface topology of fibroblasts



has revealed increased numbers of microvilli, blebs, and lamellipodia in cells at low density vs. cultured cells near confluence (17). It seems reasonable to suppose that the absolute amounts of lipid uptake by vesicle-treated cells must be related to the surface area available for vesicle-cell contact. Thus, on the basis of scanning electron microscope studies, noncontacting, low density cultures should exhibit greater lipid uptake from vesicles than confluent cultures. (b) The physical state of the plasma membrane components may be different for cells at different densities in culture. Recent studies with nontransformed mouse 3T3 cells indicated that the intramembranous particles found by freeze-fracture techniques are in an aggregated state for cells in contact with each other and in a relatively dispersed state for sparse cell cultures (21). Thus, the entry of the exogenously applied vesicle lipids may also be regulated by this difference in the membrane physical state. (c) A third possibility is that the intracellular cyclic nucleotide level changes with the culture age; low cyclic AMP and high cyclic GMP levels being associated with sparse, growing cells and vice versa (8, 12). In the present study, we demonstrate a rapid internalization and metabolism of phospholipid taken up by vesicle-treated cells. It is reasonable to assume that the rates of any metabolic process, e.g. lipid degradation, will affect the overall uptake of phospholipids. The intracellular cyclic nucleotide levels may well regulate the rates of these processes and thereby result in the apparent relationship between phospholipid uptake and density of the cell culture. Experiments designed to distinguish among the possibilities posed above are currently in progress.

While the kinetics of cell death may not precisely correlate with the appearance of trypan blue-stained cells because of some ambiguity in the staining technique, it is clear that prolonged treatment of cultured cells with lecithin vesicles leads to eventual cell death. This conclusion is based on the observation that cultures containing nearly 100% trypan blue-stained cells contained large numbers of floating cells, and on the fact that replicate plates of such cultures, untreated with dye, failed to grow or exclude dye upon addition of fresh culture medium. One possible explanation for this observation is that the maintenance of a proper membrane phospholipid/cholesterol ratio is crucial to the normal physiology of the cell, and that this ratio is drastically altered on prolonged incubation with lecithin vesicles. Indeed, we have found

that cultures, regardless of cell density, treated with 1:1 lecithin:cholesterol vesicles are 100% viable as judged by vital stains, and are indistinguishable from controls incubated in balanced salt solution alone. Additionally, large amounts of lysolecithin and free fatty acid may be produced in vesicle-treated cells during prolonged incubation, these compounds being toxic to the cells at high concentrations.

The observations that at 37°C the uptake of phospholipid from vesicles by vesicle-treated cells is inhibited by only about 25–30% in the presence of an inhibitor of respiratory electron transfer ( $\text{NaN}_3$ ) or oxidative phosphorylation (DNP), and that fixation of cells with glutaraldehyde before vesicle treatment fails to reduce the lipid uptake by more than 25–50% strongly suggest that a substantial fraction of the lipid uptake is not dependent on energy. From the data in Table II, it is seen that no effects of the metabolic inhibitors were obtained unless the carbon source, dextrose, was omitted from the incubation medium. This is consistent with the observations of others, that depletion of cellular ATP levels using such inhibitors is ineffective in the presence of glucose (28). In the following paper, we present evidence that at 37°C the predominant pathways for phospholipid uptake by vesicle-treated cells are vesicle-cell fusion and vesicle-cell lipid exchange (13). In this communication, we have shown that the normal physiological response of cultured cells to lecithin vesicle treatment is a rapid internalization of the exogenous phospholipid with some subsequent degradation. It is possible that any one, or some combination, of these processes is energy dependent. Depletion of cellular ATP levels would be expected to reduce the overall rate of lipid uptake even though an initial event such as vesicle-cell fusion or lipid exchange might not be directly affected by energy depletion.

A prerequisite for the application of electron microscope radioautography in the study of lipids is that the radioactive lipid not be selectively removed from the cell during preparation of the sample for electron microscopy. An excellent summary of the effects of various fixation procedures and dehydrating agents in relation to lipid retention in a given sample has been prepared by Stein and Stein (27). In the autoradiographic study presented here, the applied vesicle suspension was comprised of DOL to which trace amounts of [ $^3\text{H}$ ]DSL were added. Thus, the labeled phospholipid molecules contained fully saturated

fatty acids only. In lung tissue in which lecithin is present primarily as the fully saturated molecule, dipalmitoyl lecithin, it has been shown that up to 50% of the radiolabeled lecithin may be lost during sample preparation for electron microscopy, depending on fixation conditions and methods of dehydration. Thus, some loss of lipid taken up by the vesicle-treated cells can be expected in our present study. Of particular concern is that any such loss from the sample be a random one, and not a selective removal of lipid from one particular area of the cell. The autoradiographs given in Fig. 4 support a nonselective loss of label from the preparation; they are in qualitative agreement with our biochemical findings that only about 20% of the radioactivity taken up by the cells is in the plasma membrane fraction of the cell, and that the remaining lipid is found to be in subcellular fractions.

A most interesting finding of the present autoradiographic study is that the process of internalization of labeled lipid is very rapid. Thus, qualitatively, the overall distribution of label in the cell is not apparently different following either a 5-min or a 1-h treatment with labeled vesicles at 37°C. The micrographs in Fig. 4 also suggest that the distribution of label in the cytoplasm may not be entirely random, but rather that there may be a preferential accumulation of label in the cytoplasmic lipid droplets and possibly at the nuclear membrane. The existence of any significant localization of label, however, must await a complete statistical analysis of the grain distribution in the developed autoradiographs.<sup>1</sup>

The results of our electron microscope autoradiographic study and chemical analysis of vesicle-treated cells indicate that the exogenous vesicle lipid (lecithin) taken up by the cultured V79 cells examined here was rapidly internalized and eventually degraded, producing only a relatively small alteration in the plasma membrane phospholipid composition. Attempts to look for modification in the membrane properties of vesicle-treated cells showed no significant effects on the uptake of 2-deoxyglucose or the lectin-induced agglutinability of cells (Pagano, R., and L. Huang unpublished observations). Preliminary results do indicate, however, that EYL vesicle-treated cells exhibit significantly slower kinetics of adhesion to protein-coated plastic surfaces than control, un-

<sup>1</sup> Pagano, R., and L. Huang. Manuscript in preparation.

treated cells, or cells treated with 1:1 EYL/cholesterol mixed lipid vesicles. Detailed studies to examine the problem of membrane lipid alteration using other cell types (lymphocytes and muscle cells) and other lipid classes in which a slower internalization and degradation of the exogenously supplied lipids may take place are currently in progress.

The present study bears directly on the problem of using lipid vesicles, into which appropriate macromolecules have been reconstituted, to transplant a foreign membrane protein into the plasma membrane of an intact, host cell. The fate of the reconstituted vesicle protein may or may not parallel that of the exogenous lipid taken up by vesicle-treated cells. However, since 20% of this lipid remains in the plasma membrane following vesicle treatment, it is expected that some of the reconstituted vesicle protein will also stay associated with the cell surface. Depending on the proper choice of the reconstituted membrane protein, a significant modification in the plasma membrane properties and, perhaps, in the physiology of the cell can be expected.

We wish to express our appreciation to Ms. E. Asch and Mr. W. Duncan for excellent technical assistance, and to Dr. A. Amsterdam (Weizmann Institute of Science) for demonstration of the autoradiographic technique.

Leaf Huang is the recipient of a fellowship from Carnegie Institution of Washington.

Received for publication 24 January 1975, and in revised form 16 May 1975.

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