INTERACTION OF PHOSPHOLIPID VESICLES WITH CULTURED MAMMALIAN CELLS

II. Studies of Mechanism

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ABSTRACT

The mechanism of interaction of artificially generated lipid vesicles (~500 Å diameter) with Chinese hamster V79 cells bathed in a simple balanced salt solution was investigated. The major pathways of exogenous lipid incorporation in vesicle-treated cells are vesicle-cell fusion and vesicle-cell lipid exchange. At 37°C, the fusion process is dominant, while at 2°C or with energy depleted cells, exchange of lipids between vesicles and cells is important.

The fusion mechanism was demonstrated using vesicles of [^{14}C]lecithin containing trapped [^{3}H]inulin. Consistent with a fusion hypothesis, both components became cell associated at 37°C in nearly the same proportions as they were present in the applied vesicles. Additional arguments in favor of vesicle-cell fusion and against phagocytosis or adsorption of intact vesicles are presented. At 2°C or with inhibitor-treated cells, the [^{3}H]inulin uptake was largely suppressed, while the lipid uptake was reduced to a lesser extent.

Evidence for vesicle-cell lipid exchange was obtained using V79 cells grown on ^{3}H precursors for cellular lipids. [^{14}C]lecithin vesicles, incubated with such cells, showed no change in their elution properties when subjected to molecular sieve chromatography on Sepharose 4B. However, radioactivity and thin-layer chromatographic analyses revealed that a variety of cell lipids had been exchanged into the unilamellar vesicles.

Further evidence for the fusion and exchange processes was obtained using vesicles prepared from mixtures of [^{3}H]lecithin and [^{14}C]cholesterol.

A two-step fusion mechanism consistent with the present findings is proposed as a working model for other fusion studies.

The use of artificially generated lipid vesicles (liposomes or phospholipid dispersions) has recently been reported by several laboratories for producing a variety of modifications in the physiology of mammalian cells. These include the use of vesicles as carriers to cells of entrapped materials (1, 7, 12, 15, 16, 21, 24), promoting agents for cell fusion (13, 17), and as tools for modifying the lipid composition of the membranes of intact cells (2, 6, 10). The possible molecular mechanisms by which lipid vesicles produce their varied effects, however, have not been fully determined. Furthermore, many of the studies have been complicated by factors which make a mechanistic interpretation difficult if not impossible. For example, some studies on drug entrapment have been carried out in whole animals or in serum-containing medium; conditions which could favor the reorganization of

the lipid vesicle into some other structure. Others have utilized lipid vesicles prepared by mechanical dispersion of a mixed lipid system in an aqueous phase; a technique which is known to produce a heterogeneous collection of multicompartmented structures of widely varying size and shape.

In the accompanying paper (9), we presented our observations on the characteristics of the uptake of phospholipid by cultured cells upon incubation, in a simple balanced salt solution, with chemically and physically well-defined unilamellar lipid vesicles. In this paper, detailed studies on the mechanism of this uptake are presented. A preliminary report of these findings has appeared elsewhere (14).

MATERIALS AND METHODS

The techniques for culturing the Chinese hamster V79 cells used in these studies were identical to those given in the preceding paper (9).

L-α-di[1-^{14}C]oleyl phosphatidyl choline was synthesized as described previously (9), using [1-^{14}C]oleic acid. Radioactively labeled cholesterol, deoxyglucose, inulin, oleic acid, and sucrose were obtained from New England Nuclear, Boston, Mass.

Studies of Vesicle-Cell Fusion

Experiments designed to test the existence of a vesicle-cell fusion mechanism utilized unilamellar vesicles generated from [^{14}C]dioleyl lecithin (DOL) in which a suitable ^{3}H marker was sequestered. Such vesicles were generated by cosonication of 100 mg [^{14}C]DOL (5 μCi) with 1 mCi of carrier free [^{3}H]inulin (500 mCi/gm) or [^{3}H]sucrose (500 mCi/mmol) in 4 ml of a modified Gey's balanced salt solution in which the carbon source, dextrose, was omitted. Sonication was carried out for 1 h at 2°C in an argon atmosphere as described in the preceding paper (9). In order to separate the population of unilamellar vesicles containing the trapped marker from multilamellar vesicles and from untrapped [^{3}H]inulin or sucrose, the sonicated lipid dispersion was subjected to gel filtration chromatography on a Sepharose 4B column (2.5 × 40 cm) (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The column was eluted with modified Gey's, and the absorption of the column effluent at 280 nm was continuously recorded with a Gilson UV column monitor (Gilson Medical Electronics, Inc., Middleton, Wisconsin). Fractions of 2 ml were collected and refrigerated, and aliquots taken for liquid scintillation counting. All radioactivity counting data were corrected for background and channel crossover. The unilamellar vesicle fractions (nos. 35–55, Fig. 1) containing the trapped marker were pooled and divided into two equal volumes. One was diluted with modified Gey's salt solution to a final lipid concentration of 1 mg/ml, and a concentrated solution of NaN_3 was then added to make the vesicle suspension 30 mM in inhibitor. The second volume of pooled vesicles was diluted with Gey's, and concentrated dextrose added to make the suspension equivalent to normal Gey's (11 mM dextrose). These two vesicle suspensions were used in uptake experiments as described in the preceding paper (9), except that the cell cultures were preincubated (2 h at 37°C) either in modified Gey's containing 30 mM NaN_3 or in normal Gey's before the addition of the corresponding vesicle suspension. One series of experiments utilized cells which were treated with unlabeled inulin (20 mg/ml) before (1 h at 37°C) and during incubation with the [^{3}H]inulin-containing vesicles. These experiments were carried out in normal Gey's salt solution.

Additional suspensions of DOL vesicles were prepared containing trapped 5-bromo-4-chloro-3-indolyl-β-D-galactoside, a histochemical substrate for the lysosomal enzyme β-galactosidase. This material was present at a concentration of 2 mg/ml during the sonication procedure. After sonication, the lipid dispersion was subjected to molecular sieve chromatography as described above, and the pooled vesicle fractions were used in an uptake experiment. The presence of the trapped substrate was indicated by the deep blue color of the unilamellar vesicle fraction. Vesicle-treated cells were then examined in a phase-contrast microscope for color development.

Studies of Vesicle-Cell Lipid Exchange

Labeling of cell lipids in order to examine the question of molecular exchange mechanisms between phospholipid vesicles and cultured cells was carried out using cells grown in a culture medium containing radiolabeled palmitic acid (23). Approximately 10^7 cells were inoculated into each of two roller bottles containing 150 ml of culture medium and 2 mCi of carrier-free [9,10-^{3}H]palmitic acid (330 mCi/mmol). Cells were allowed to grow in this medium for 48 h at 37°C. The labeled medium was then replaced with unlabeled medium, and cell growth allowed to continue for another 24 h. The cells attached to the surface of the roller bottles were then washed extensively with Gey's and incubated for 1 h at 37°C with [^{14}C]DOL (0.05 μCi/mg) unilamellar vesicles (1 mg phospholipid/ml Gey's). After this incubation, the supernate was removed and briefly centrifuged (10,000 × g; 10 min) to remove any whole cells or fragments that were detached from the roller bottles during the course of the incubation. The supernate from this centrifugation was then concentrated to a final volume of 10 ml on an Amicon ultrafiltration device (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) using a 43-mm diameter XM-100A membrane with rapid stirring under N_2 pressure (10 lb/inch^2). The concentrated suspension was next applied to a Sepharose 4B column (2.5 × 40 cm) and the effluent monitored by absorbance measurements at 280 nm, and determinations of ^{14}C cpm and ^{3}H cpm. Samples of interest (see Results) were pooled, extracted with an equal volume of 2/1:chloroform/methanol, and the organic phase was taken to dryness under N_2. These

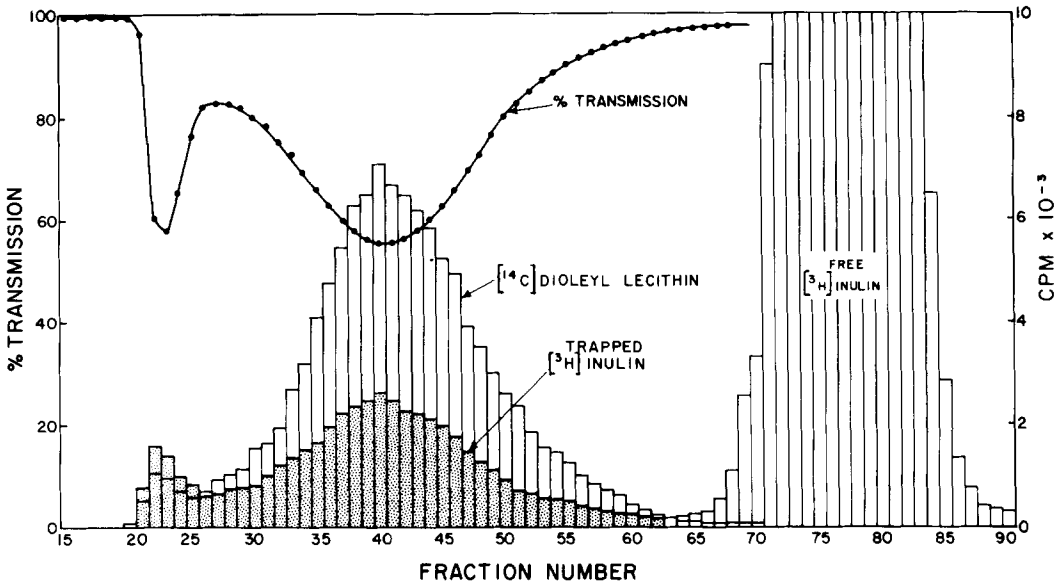

FIGURE 1 Elution profile of sonicated dispersion of [^{14}C]DOL and [^{3}H]inulin chromatographed on a 2.5 × 40 cm Sepharose 4B column, showing percent transmission at 280 nm and profile of ^{14}C cpm and ^{3}H cpm.

extracted lipids were dissolved in a small volume of 2/1:chloroform/methanol and spotted in one corner of a precoated silica gel plate (20 × 20 cm). A two-dimensional, thin-layer chromatograph was then developed using the following solvent systems: (*a*) chloroform-methanol-28% ammonia (65:25:5, vol/vol) and (*b*) chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1, vol/vol). The final developed plate was visualized either with I_2 vapors or by autoradiography using X-ray film. In the former, the individual spots were scraped from the plate and processed for liquid scintillation counting.

In control experiments, the cells grown in identical roller bottles were incubated with Gey's (containing no vesicles) for 1 h at 37°C. The solution was then removed from the bottles, centrifuged at 10,000 × g for 10 min, and used in subsequent incubations (1 h, 37°C) either with Gey's alone or with a second batch of identically prepared unilamellar [^{14}C]DOL vesicles. After these incubations, the solutions were processed for column chromatography and thin-layer chromatographic (TLC) analysis as described above.

Lecithin-Cholesterol Vesicles

Mixed lipid vesicles containing [^{3}H]DOL (5.7×10^{-13} cpm/molecule) and [^{14}C]cholesterol (1.5×10^{-12} and 0.5×10^{-12} cpm/molecule for 22% and 46% cholesterol, respectively) were prepared by premixing the desired quantities of these materials in an organic solvent and evaporating to dryness under N_2 before suspension in Gey's and sonication. Vesicles were subsequently chromatographed on Sepharose 4B as described above; only unilamellar fractions were used in uptake experiments. For the studies reported in this paper, two different mole fractions of cholesterol in lecithin were utilized (0.22 and 0.46). In both cases the ratio, ^{14}C cpm/^{3}H cpm, of the pooled vesicle fractions was unity.

RESULTS

Vesicle-Cell Fusion

Fig. 1 shows an elution profile of lecithin vesicles chromatographed on Sepharose 4B. The vesicles were prepared by cosonication of [^{14}C]DOL in modified Gey's (see Materials and Methods) containing [^{3}H]inulin as a marker for the internal aqueous space of the vesicles. The profile of percent transmission vs. fraction number clearly shows the existence of multilamellar (fractions 20–25) and unilamellar (fractions 30–60) vesicles (8). From the ^{14}C cpm, it is seen that the unilamellar vesicles represent 95% or more of the total lipid eluted from the column. The profile of ^{3}H cpm shows that the free untrapped inulin (fractions 65–90) was well separated from the vesicle fractions by chromatography on Sepharose 4B. The ratio, ^{14}C cpm/^{3}H cpm, in the pooled unilamellar vesicles was 3.2. No change in this ratio was detected after 24 h, when the vesicles were passed over the Sepharose column a second time. Thus, it is concluded that no leakage of inulin occurred

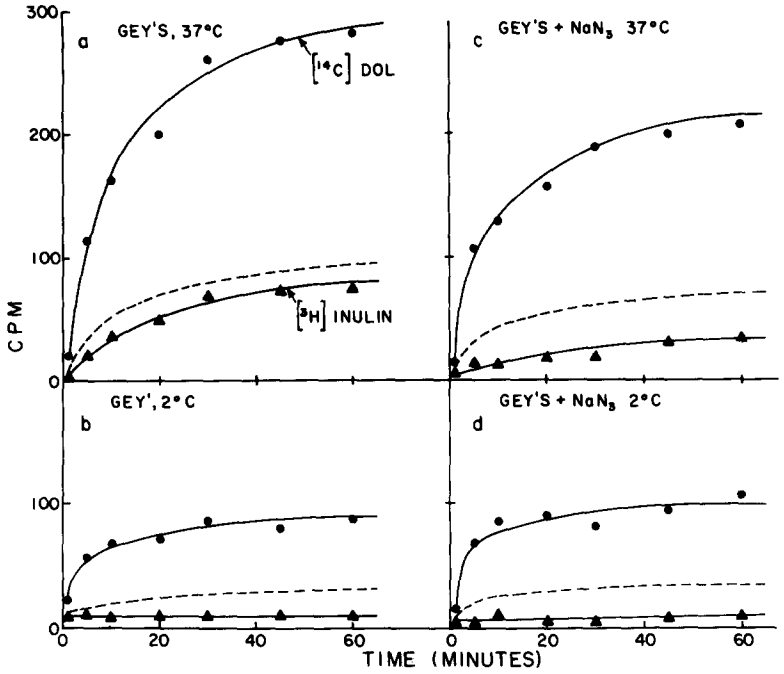

FIGURE 2 Incubation of Chinese hamster V79 cells with unilamellar lipid vesicles generated from [^{14}C]DOL and containing sequestered [^{3}H]inulin. The ratio, ^{14}C cpm/^{3}H cpm, for all samples of applied vesicles was 3.2. Dashed curve represents the expected amount of [^{3}H]inulin uptake calculated from the observed [^{14}C]lipid uptake and the ^{14}C cpm/^{3}H cpm ratio of the applied vesicles. (*a* and *b*) incubation at 37°C and 2°C in Gey's balanced salt solution. (*c* and *d*) incubation at 37°C and 2°C using energy-depleted cells (see text) in the presence of 30 mM NaN_3. The ordinate is the observed cpm per 10^7 cells.

before the time the vesicles were used in actual uptake experiments. This was also true when sucrose was the trapped marker.

The results of two separate uptake experiments at 2°C and 37°C in which such doubly-labeled vesicles and V79 cells were used are shown in Fig. 2. Each panel of this figure shows the observed uptake of [^{14}C]DOL and [^{3}H]inulin by vesicle-treated cells. In Fig. 2 *a* and *b*, cells were incubated with a vesicle suspension made up in Gey's. In Fig. 2 *c* and *d*, the incubation of cells with vesicles was carried out in modified Gey's containing 30 mM NaN_3. The precise conditions of energy depletion are given in the Materials and Methods section. The dashed line in each curve represents the theoretical amount of [^{3}H]inulin that should have become cell associated had both the vesicle lipid and trapped marker entered the cell together. This line was calculated from the *observed* uptake of [^{14}C]DOL and the ratio, ^{14}C cpm/^{3}H cpm (= 3.2), in the applied vesicle suspensions. The data for lipid uptake confirm the observations given in the preceding paper (9), namely, the uptake of lipid from unilamellar vesicles by V79 cells was inhibited by about 25% at 37°C under conditions of energy depletion, whereas at 2°C no effect of inhibitor on the lipid uptake could be detected. At 2°C, in both the presence (Fig. 2 *d*) and the absence (Fig. 2 *b*) of inhibitor, relatively little inulin uptake was observed in a 1-h period, and this was low compared to the uptake expected on the basis of lipid incorporation. However, incubations with vesicles at 37°C in Gey's (Fig. 2 *a*) showed that the inulin uptake was almost 90% of the expected value, while for energy-depleted cells (Fig. 2 *c*) about 45% of the expected value was obtained. Treatment of cells with unlabeled inulin before and during the actual incubation with [^{3}H]inulin-containing vesicles had no effect on the amounts of [^{3}H]inulin that became cell associated during vesicle treatment. This excludes the possibility that free [^{3}H]inulin was released from the vesicles and adsorbed to the outer surface of the cells.

Experiments in which [^{3}H]sucrose was used as the trapped marker showed that only about 15% of the expected sucrose became cell associated during vesicle treatment at 37°C in Gey's.

Cells preloaded with [^{3}H]deoxyglucose (18) and subsequently treated with either egg yolk lecithin or DOL vesicles showed no detectable leakage of the labeled, water-soluble metabolite during the incubation with vesicles.

No histochemical stain was observed in cells treated for 1 h at 37°C with DOL vesicles containing a trapped substrate for the lysosomal enzyme, β-galactosidase.

Vesicle-Cell Lipid Exchange

The method of labeling cell lipids by growth on radioactive palmitic acid (23) was found to be very satisfactory for the Chinese hamster cell line used in these studies. Almost all the radiolabeled material in the cell was chloroform-methanol extractable, indicating that only lipids were labeled under the conditions employed in this study. Thin-layer chromatographs obtained for chloroform-methanol extracts of cells grown on [^{3}H]palmitic acid showed that many of the cellular glycerolipids and phospholipids had become radiolabeled, and that little, if any, free palmitic acid remained.

Fig. 3 shows the results of an experiment in which cells grown on [^{3}H]palmitic acid were subsequently incubated with [^{14}C]DOL vesicles. In Fig. 3 *a*, the elution profile of the unilamellar vesicle fraction before interaction with cells is shown. It is seen to consist of a broad symmetrical peak, both in absorbance and in ^{14}C cpm, centered about fraction number 36. Fig. 3 *b* shows the profiles of absorbance at 280 nm, ^{3}H cpm and ^{14}C cpm for a suspension of [^{14}C]DOL vesicles following interaction with the ^{3}H-labeled cells. The profile of absorbance consists of four distinct peaks. The first peak, corresponding to the column void volume, represents a material of high molecular weight ($\geq 20 \times 10^6$ daltons), e.g. cellular fragments or lipoprotein aggregates, which was not removed from the vesicle suspension by low speed centrifugation. The second peak, centered approximately at fraction 36, represents the unilamellar lipid vesicles. Both peaks are seen to contain ^{3}H cpm and ^{14}C cpm. The remaining two peaks of absorbance have no detectable amounts of radioactivity associated with them and probably correspond to soluble proteins. In the control experiment in which the lecithin vesicles were not incubated directly with the cells, but rather with a simple salt solution which previously was in contact with the ^{3}H-labeled cells, a similar profile of absorbance vs. fraction number was obtained (Fig. 3 *c*). While the first two major peaks are again seen to contain both ^{3}H and ^{14}C, the relative amounts of these are considerably different from those given in Fig. 3 *b*. Results qualitatively similar to those presented in Fig. 3 were also obtained with cells whose lipids were radiolabeled by growth on [2-^{3}H]glycerol (4).

Fractions 35–48 from the column represented by the profile in Fig. 3 *b* were pooled, and the lipids subsequently extracted with 2/1:chloroform/methanol. Analysis of the extracted lipids by TLC revealed six major spots (A–F, Fig. 4). Spot C was identified as phosphatidyl choline, and F corresponded to cholesterol and other less polar lipids, e.g. triglycerides. The remaining spots were not firmly identified but are most probably lyosphosphatidyl choline (spot A), sphingomyelin (spot B) and glycolipids (spots D and E). A similar two-dimensional chromatograph was obtained from the lipids extracted from pooled fractions 34–47 of the column represented in Fig. 3 *c*.

The spots from each thin-layer plate were subsequently scraped from the TLC plate and analyzed for both ^{3}H and ^{14}C. The results of this analysis are given in Table I. It is seen that all spots from both plates contain ^{3}H and ^{14}C, with the possible exception of the less polar lipids, spot F, which is very high in ^{3}H cpm and contains practically no ^{14}C. Furthermore, it is seen that the ratio ^{14}C cpm/^{3}H cpm of spots A–F varies considerably. If the ratio of the total ^{14}C cpm to total ^{3}H cpm scraped from each plate is made, it is seen that this number (0.40 for TLC-I, and 1.22 for TLC-II) agrees well with the corresponding ^{14}C cpm/^{3}H cpm ratio of the samples which were applied to each plate (0.39 for TLC-I and 1.29 for TLC-II). These numbers also agree well with the ratios of the pooled samples before chloroform-methanol extraction, indicating that no preferential extraction of the lipids occurred.

In the control experiment in which the Gey's supernate (containing no [^{14}C]DOL vesicles) was chromatographed on Sepharose 4B, a small constant abosrbance (~0.035 OD units) was found over the fractions corresponding to the unilamellar vesicles. TLC analysis of the chloroform/methanol-extractable materials from these fractions revealed the presence of less polar lipids only.

The uptake of V79 cells of DOL and cholesterol

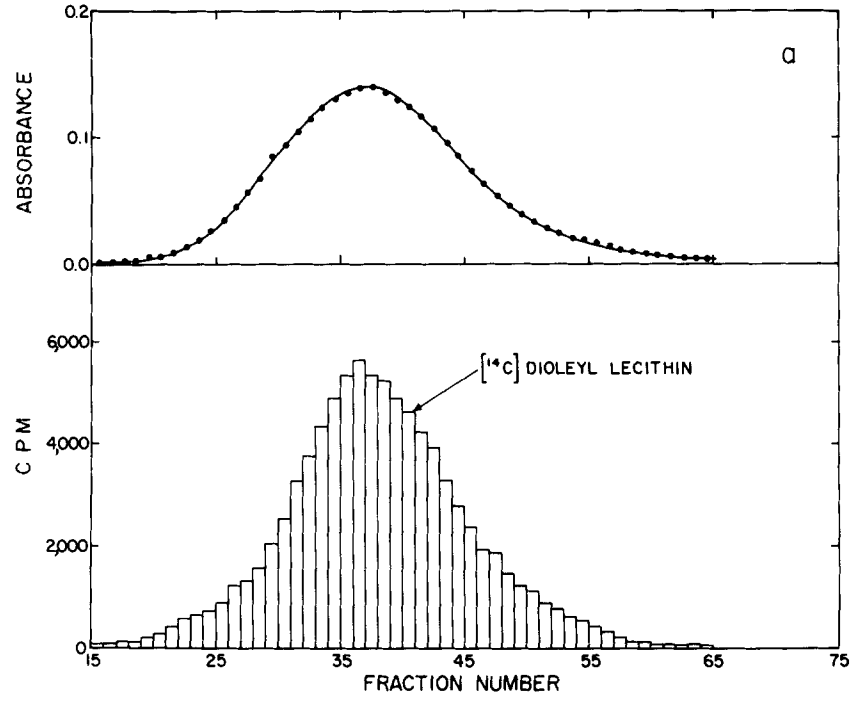

FIGURE 3 *a*

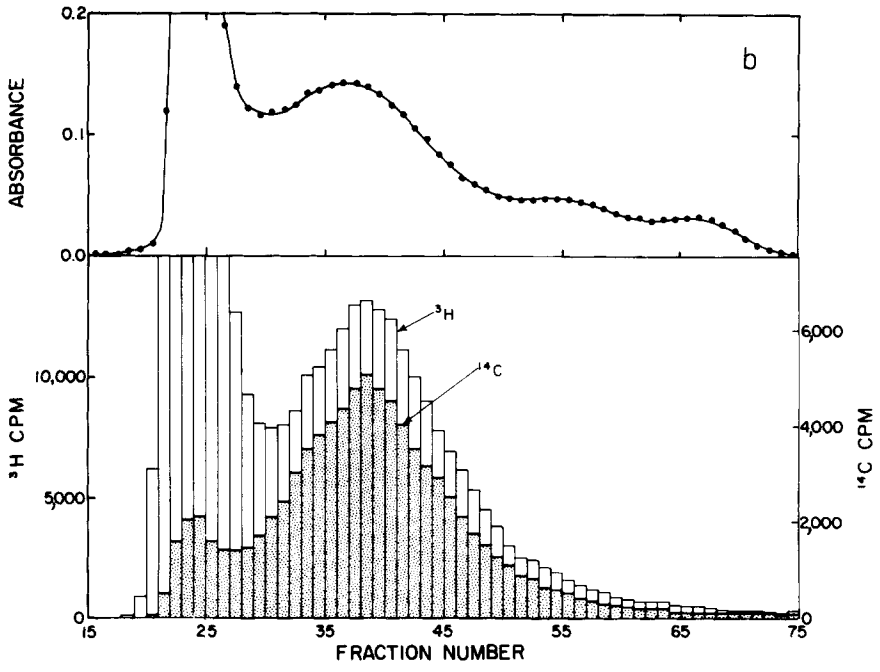

FIGURE 3 *b*

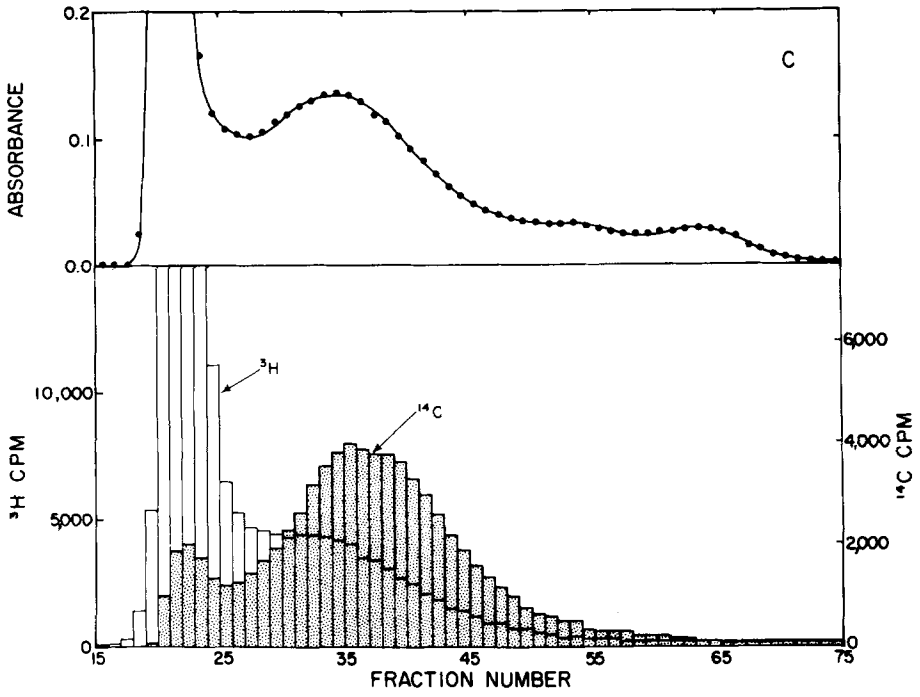

FIGURE 3 *c*

FIGURE 3 (*a*) Elution profile of unilamellar fraction of [^{14}C]DOL vesicles chromatographed on Sepharose 4B (2.5 × 40 cm). Absorbance at 280 nm, and ^{14}C cpm vs. fraction number. (*b*) Elution profile of unilamellar [^{14}C]DOL vesicles following a 1-h incubation at 37°C with Chinese hamster V79 cells. Cell lipids were labeled with ^{3}H by growth on [^{3}H]palmitic acid. Absorbance at 280 nm, ^{3}H cpm and ^{14}C cpm are plotted vs. fraction number. In (*c*), cells were incubated for 1 h at 37°C in Gey's containing no vesicles. The supernate was then used for a 1-h incubation at 37°C with [^{14}C]DOL vesicles, and chromatographed on Sepharose 4B as in (*b*).

from vesicles containing a mixture of these two components has also been examined. The results of uptake experiments, both at 2°C and at 37°C, and for two different mole fractions of cholesterol (0.22 and 0.46) are given in Fig. 5. In each experiment the ratio, ^{14}C cpm/^{3}H cpm, in the applied vesicle suspension was unity. One essential characteristic of the uptake process using such mixed vesicles is that, at 2°C, lecithin preferentially becomes cell associated relative to cholesterol. At 37°C, the two components become cell associated in the same proportions as they exist in the applied vesicles. This observation is a general one, holding true over all cholesterol-lecithin mole fractions examined (0.1–0.5).

DISCUSSION

The experiments presented in this paper were designed to determine the relative contributions of the various pathways that can exist in the interaction between a uniform suspension of chemically and physically well-defined lipid vesicles with cultured mammalian cells.

Adsorption and Phagocytosis of Intact Vesicles

Adsorption of intact vesicles to the surface membrane of cultured cells was excluded on the basis of the following. First, introduction of either a positive or negative surface charge into vesicles by cosonication of radiolabeled lecithin with 10% stearyl amine (positively charged), 10% phosphatidyl glycerol, or 10% phosphatidyl serine (both negatively charged) does not affect the amount of radiolabeled lecithin that becomes cell associated (9). This suggests that any surface adsorption of intact vesicles due to electrostatic cell-vesicle in-

teractions is unimportant. Secondly, in transmission electron micrographs of thin sections through vesicle-treated cells, no attached vesicles were ever observed on the cell surface, nor was radiolabeled lipid localized exclusively on the cell surface in electron microscope autoradiographs (9).

The possibility that intact vesicles are being engulfed by vesicle-treated cells in a process resembling phagocytosis is excluded on the basis of the following. (*a*) In thin sections of vesicle-treated cells, no unilamellar vesicles were ever seen inside any structures resembling phagosomes. (*b*) Electron microscope autoradiographs show that the radioactive lipid, which becomes internalized in the cell, is distributed throughout the cytoplasm of the cell and *not* confined to structures like phagosomes (9). This was found to be true for very brief incubation periods (5 min) as well as for incubations carried out in the presence of inhibitors of energy metabolism. (*c*) If the applied vesicles were phagocytosed, they should have been delivered to the lysosomal apparatus of the cell and subjected to hydrolysis and digestion. Attempts to demonstrate such a possibility using vesicles containing trapped 5-bromo-4-chloro-3-indolyl-β-D-galactoside, a sensitive histochemical substrate for the lysosomal enzyme, β-galactosidase, failed. Had this material been subjected to hydrolysis by the lysosomal glucosidase, it would have been converted to an insoluble and intensely chromogenic compound which is readily detectable at the sites of enzymatic activity (19).

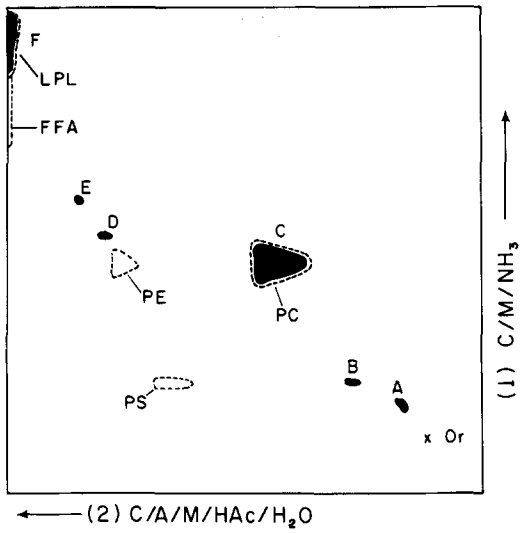

FIGURE 4 Two-dimensional thin-layer chromatograph on silica gel of lipids extracted from ^{14}C-labeled vesicles which were incubated with ^{3}H-labeled cells (Fig. 3 *b*; fractions 35–45). Chromatographs were developed in solvent systems (1) Chloroform-methanol-28% ammonia (65:25:5, vol/vol) and (2) chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1, vol/vol). Six major spots were observed (A–F); the dashed lines show the position of known standards (*PC* = phosphatidyl choline; *PS* = phosphatidyl serine; *PE* = phosphatidyl ethanolamine; *FFA* = free fatty acids; *LPL* = less polar lipids, e.g., cholesterol, triglycerides, etc.) developed under identical conditions. *Or* = origin.

Vesicle-Cell Fusion

If a fusion process is involved in the interaction of phospholipid vesicles with cultured cells, then the aqueous contents of such vesicles should be released into the cytoplasm of vesicle-treated cells following each fusion event. In order to test this possibility, vesicles comprised of [^{14}C]DOL and containing [^{3}H]inulin as a marker for the internal aqueous space of the vesicles were prepared and used in uptake experiments with V79 cells (Fig. 2). From these data we can exclude any significant amount of vesicle-cell fusion at 2°C, whereas, at 37°C, roughly 90% of the phospholipid uptake can be accounted for by a vesicle-cell fusion mechanism.

To rigorously demonstrate the existence of a fusion mechanism it must be shown, in addition to the uptake of the labeled phospholipid and trapped marker, that the latter, once it becomes cell associated, is free within the cytoplasm of the cell. To test this notion, we have attempted to trap a variety of electron-dense markers within vesicles and visualize their location within vesicle-treated cells by electron microscopy. However, it was impossible to successfully trap, by sonication or injection, any of the markers tried (ferritin, iron-dextran, thorium sol, polyglutamic acid-Pb complex). The use of histochemical stains suitable for electron microscopy such as horseradish peroxidase was considered, but the resolution of this technique is insufficient to unequivocally demonstrate fusion of ~ 500 Å diameter vesicles with cells. Papahadjopoulos et al. (15, 16) found that cultured mammalian cells treated with cyclic-AMP-containing vesicles showed a marked reduction in their growth rates. This result was interpreted in terms of a vesicle-cell fusion process; however, direct analytical determination of the soluble, cytoplasmic cyclic-AMP levels was not provided. Thus, while fusion of lipid vesicles with cells is the most consistent explanation we can

TABLE I

Radioactive Analysis of Two-Dimensional Thin-Layer Chromatograms of Lipids Extracted From [^{14}C]DOL Vesicles Following Their Incubation With ^{3}H-Labeled Chinese Hamster V79 Cells.

Spot	^{3}H cpm	^{14}C cpm	^{14}C cpm/ ^{3}H cpm
TLC-I: Vesicles incubated directly with cells			
Or	34	28	0.8
A	45	122	2.7
B	918	33	0.04
C	3,807	5,559	1.5
D	96	605	6.3
E	28	68	2.4
F	11,124	19	0.002
Total from plate:	16,052	6,434	0.40
Applied to plate:	—	—	0.39
Pooled samples, before extraction:		—	0.36
TLC-II: Vesicles incubated with supernate (see text)			
Or	12	14	1.2
A	32	97	3.0
B	281	41	0.1
C	3,237	6,172	1.9
D	198	964	4.9
E	47	105	2.2
F	2,257	16	0.007
Total from plate:	6,064	7,409	1.22
Applied to plate:	—	—	1.29
Pooled samples, before extraction:		—	1.19

offer for our observations on vesicle-cell interactions, as well as observations obtained from other laboratories, direct unambiguous proof that the trapped marker, which becomes cell associated upon vesicle treatment, is truly free in the ctyoplasm is still missing.

Vesicle-Cell Lipid Exchange

The data discussed in the preceding section indicate that, at 37°C, about 90% of the phospholipid uptake by vesicle-treated cells can be accounted for by a fusion mechanism. The results in Fig. 3 suggest that an additional pathway, lipid exchange, may account for the remaining lipid uptake. This conclusion is based on the molecular sieve properties and radiotracer analysis of the [^{14}C]DOL vesicles before and after incubation with the ^{3}H-labeled cells. Following vesicle-cell incubation, some of the [^{3}H]cell lipids were observed to precisely cochromatograph with the peak of [^{14}C]lipid vesicles (Fig. 3 *b*). Furthermore, the position of this peak corresponded quantitatively to its position in the original chromatograph obtained before incubation with the cultured cells (Fig. 3 *a*). These observations suggest that during the course of vesicle-cell incubation some of the cell lipids become an integral part of the phospholipid vesicles, with no significant change in vesicle size. This can best be explained by an exchange process in which lipid molecules from the cell surface and vesicle are interchanged. The data in Fig. 3 *c* show that, when lipid vesicles are incubated with a balanced salt solution containing the lipids and proteins normally given off by cells placed in a protein-free medium (20), a smaller fraction of the cell lipids becomes associated with the lipid vesicles. Thus, the contribution of exchange between lipids in the cell supernate and the vesicles is small relative to the vesicle-cell lipid exchange.

If an exchange process is involved in the interaction of lipid vesicles with cultured cells, it is reasonable to assume that, in vesicles comprised of several lipid components, these components might exhibit different rates of exchange with the cell membrane lipids. The use of mixed vesicles comprised of [^{14}C]cholesterol and [^{3}H]lecithin showed, however, that regardless of the mole fraction of

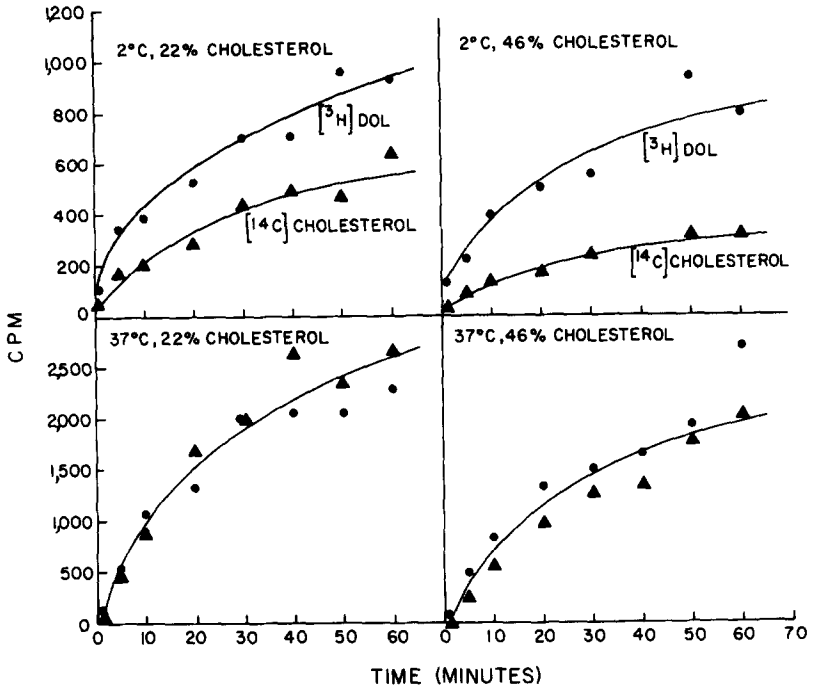

FIGURE 5 Uptake of [^{3}H]DOL (●—●) and [^{14}C]cnolesterol (▲—▲) by V79 cells from mixed vesicles, containing either 22 or 46 mole % cholesterol. The ratio ^{3}H cpm/^{14}C cpm in the applied vesicle suspension was 1.0. The ordinate is the observed cpm per 10^7 cells.

cholesterol in lecithin, the two components entered the cell at the same rate when the vesicle-cell incubation was carried out at 37°C. Since only about 10% of the lipid uptake at 37°C can be accounted for by an exchange mechanism, while the remainder is most likely due to vesicle-cell fusion, this finding is not surprising. At 2°C, where fusion is largely suppressed, the differential exchange of lipids between vesicles and cells becomes important, with cholesterol entering the cell more slowly than lecithin.

The actual mechanism of the vesicle-cell lipid exchange demonstrated here remains to be elucidated. The exchange process might be the result of one or more of the following: (*a*) A physical transfer of lipid components between membranes when the cell and vesicle come into momentary contact, (*b*) an exchange mechanism involving an enzymatic transfer of acyl chains from the exogenously supplied lecithin to other plasma membrane lipids, and vice versa, or (*c*) a carrier mechanism involving specific phospholipid exchange proteins (25) which could transfer lipids between the vesicles and cells. These possibilities will be explored in future studies.

A Proposed Fusion Mechanism

The results of our present studies on the mechanism of interaction of lipid vesicles with cultured cells have led us to consider a two step mechanism for vesicle-cell fusion as given in Fig. 6. This proposed mechanism can account simultaneously for both vesicle-cell fusion and some vesicle-cell lipid exchange. In this scheme, when a lipid bilayer vesicle collides with the plasma membrane of the cell (Fig. 6 *a*), an intermediate structure (Fig. 6 *b*) is formed in which the outer monolayer of vesicle lipids becomes an integral part of the outer monolayer of the plasma membrane lipid bilayer, with the aqueous interior of the vesicle and its surrounding inner monolayer of lipids splitting or dividing the bilayer of the plasma membrane. A similar type of structure has been postulated (5) to explain the penetration of echinoderm eggs by oil droplets (3, 11). The driving force for generation of the intermediate structure shown in Fig. 6 *b* would be the reduction in free energy of the system by elimination of the relatively high energy lipid bilayer vesicle/water interface, and its replacement with a lower energy cytoplasm/intermediate/

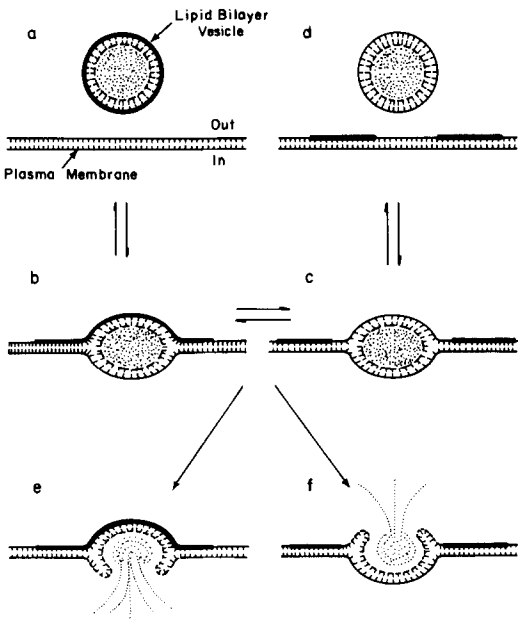

FIGURE 6 Schematic diagram of proposed mechanism for interaction of unilamellar vesicles with the plasma membrane of a cultured cell at 37°C. The outer and inner monolayers of vesicle lipids are represented, respectively, by a heavy black line and a thin dashed line. (*a*) Lipid bilayer vesicle containing sequestered material collides with the cell membrane to form an intermediate structure as given in (*b*). In this structure, the outer monolayer of lipids from the vesicle may mix with the outer monolayer of lipids in the plasma membrane to give the intermediate structure shown in (*c*). Either intermediate (*b* or *c*) can revert to an intact vesicle, (*a* or *d*), or rupture as shown in (*e* or *f*). Rupture toward the cell interior would be recorded as a fusion event (*e*), whereas rupture toward cell exterior (*f*) would transfer all the vesicle lipids to the cell without any transfer of the trapped contents. Rupture is depicted only for the intermediate structure shown in (*b*).

water interface. During the lifetime of the intermediate, the outer monolayer vesicle lipids would be free to diffuse laterally in the plane of the plasma membrane and intermix with the cell lipids as depicted in Fig. 6 *c*. The intermediate structures in Fig. 6 *b* and *c* could pinch off to give intact vesicles containing either the original complement of vesicle lipids (Fig. 6 *a*) or containing both cell lipids and vesicle lipids (Fig. 6 *d*). The latter case would be measured as a molecular exchange event. According to this scheme, the cell lipids would be confined to the outer monolayer of the newly generated bilayer vesicle. The intermediate structures (Fig. 6 *b* or *c*) could also rupture. If the rupture occurs toward the cytoplasm (Fig. 6 *e*), the discharge of the vesicular aqueous contents would be registered as a fusion event, and the vesicle lipids would be added symmetrically to both sides of the plasma membrane. Such a process might be mediated by an enzymatic reaction, e.g., a phospholipase activity in the cytoplasm. If the intermediate structures rupture toward the outside, the result would be no association of the trapped contents with the cell, and addition of both inner and outer monolayer vesicle lipids to the outer monolayer of the plasma membrane of the cell.

The proposed mechanism is consistent with the following experimental observations of vesicle-cell interactions. First, it was observed that while small molecules such as sucrose can leak out of the *vesicle* during its interaction with cells, no simultaneous leakage of metabolite (e.g. [^{3}H]deoxyglucose) from the *cell* occurs. This is contrary to observations of natural membrane fusions which have been demonstrated to be leaky processes (18, 22). This discrepancy can be explained in terms of the proposed model by requiring that any transient leak formed during vesicle-cell fusion, occurs during the formation of the intermediate structure (Fig. 6 *b*), and not during actual discharge of the vesicular contents into the cytoplasm of the cell. A second observation, given in an earlier report (14), demonstrating lack of a fusion of large ($\geq$ 1,500 Å) multilamellar vesicles with cells, is also consistent with the proposed model. We argue that the intermediate structure (Fig. 6 *b*) is unable to form in the case of the large vesicles due to their relatively low surface energy.

It should eventually be possible to further test the scheme proposed in Fig. 6 if electron microscope techniques of sufficient resolution directly reveal the existence of the postulated intermediate structures. The use of vesicles that have an asymmetric distribution of radiolabeled lipid may also prove useful. In the interim, it is hoped that the results presented in this study will prove beneficial, not only in dealing with the cellular modification phenomena produced by lipid vesicles, but also in considering natural membrane fusion processes.

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