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LIPOSOMES INJECTED INTRAVENOUSLY INTO MICE ASSOCIATE WITH LIVER MITOCHONDRIA

AMELIA CUDD, HENRI LABBE, MONIQUE GERVAIS and CLAUDE NICOLAU *

Centre de Biophysique Moléculaire du CNRS, 1A, Avenue de la Recherche Scientifique, 45045 Orléans Cédex (France)

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Liposomes encapsulating uranyl acetate or ferritin were injected intravenously into mice. At periods of 20 min, 1 h and 4 h post-injection, animals were killed, and livers were excised. Transmission electron micrographs of liver tissue showed association of oligolamellar liposomes with mitochondria for each time period. At 1 h post-injection, an average of one out of ten mitochondria was associated with liposomes. In most cases, the liposomes were clearly enclosed in a cytoplasmic vacuole. Phagocytosis by Kupffer cells as well as fusion with primary lysosomes and inclusion in secondary lysosomes was observed. No difference in intracellular fate was observed when lactosylceramide was incorporated in the liposome bilayers, suggesting that the differences observed in biochemical studies are at the level of liposome-plasma membrane interaction. When liposomes containing uranyl acetate were intravenously injected and hepatocytes were isolated by collagenase perfusion one hour later, transmission EM revealed the presence of liposomes in these cells, in cytoplasmic vacuoles in the cytoplasm and in association with mitochondria. A freeze-fracture-etching analysis of liver tissue excised 20 min after injection of liposomes encapsulating ferritin, further supported the observation that liposomes associate with mitochondria in the liver.

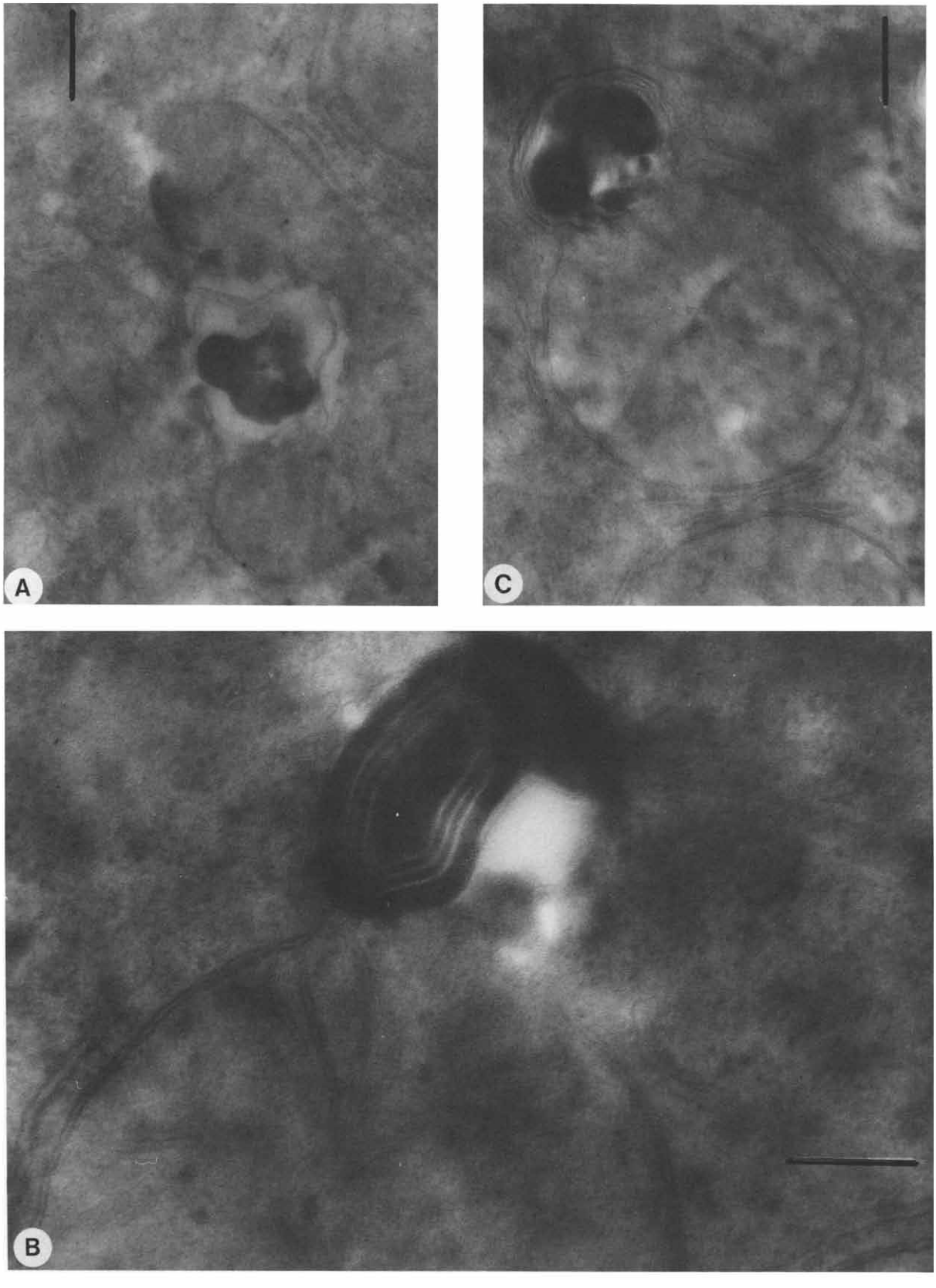
Introduction

Liposomes have long been envisioned as carriers of drugs and macromolecules, and a considerable effort has been made to elucidate their in vivo fate [1,2]. It is generally accepted today that substantial factions of liposomes comprised of phospholipid and cholesterol, when injected intravenously into mice or rats, are rapidly cleared from the blood to localize in the liver and spleen [3–5]. In the liver, large liposomes (> 2500 Å) are primarily taken up by Kupffer cells. Smaller liposomes may pass through the fenestrae of the endothelial lining to enter the hepatocytes [1–5]. These mechanisms have been drawn largely from experiments using radiolabeled lipids and markers of the aqueous space [1–5].

Detection of the transient expression of the liposome-encapsulated rat preproinsulin I gene in the rat liver and spleen [6] raised the question concerning the intracellular fate of the DNA-loaded liposomes, intravenously injected. In an effort to visualize directly the intracellular fate of liposomes in vivo, we examined by transmission electron microscopy (transmission EM) the liver tissue of mice which had been injected intravenously with liposomes. As a liposome marker, we encapsulated the electron dense metal, uranyl acetate. In other experiments, we encapsulated

* To whom correspondence should be addressed.

Abbreviations: TEM, transmission electron microscopy; PC, phosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol; LC, lactosylceramide; PL, phospholipid.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ferritin, an electron dense protein frequently used as a label in transmission EM [7] and recently used as a liposome marker in in vitro studies [8,9]. Further, a freeze-fracture-etching analysis was performed on liver tissue excised 20 min after intravenous injection of liposomes containing ferritin.

The results of these ultrastructural studies are described here. It is demonstrated that liposomes injected intravenously are later found enclosed in cytoplasmic vacuoles and in association with mitochondria in the liver.

Materials and Methods

Preparation of liposomes. Liposomes comprised of egg yolk phosphatidylcholine (PC), ox brain phosphatidylserine (PS) and cholesterol (Chol) (4 : 1 : 5, mol/mol) were prepared by reverse phase evaporation (REV) [10]. Some preparations of liposomes included lactosylceramide (LC) (LC/phospholipid (PL) 1 : 10 mol/mol), in the bilayers. All lipids were from Sigma.

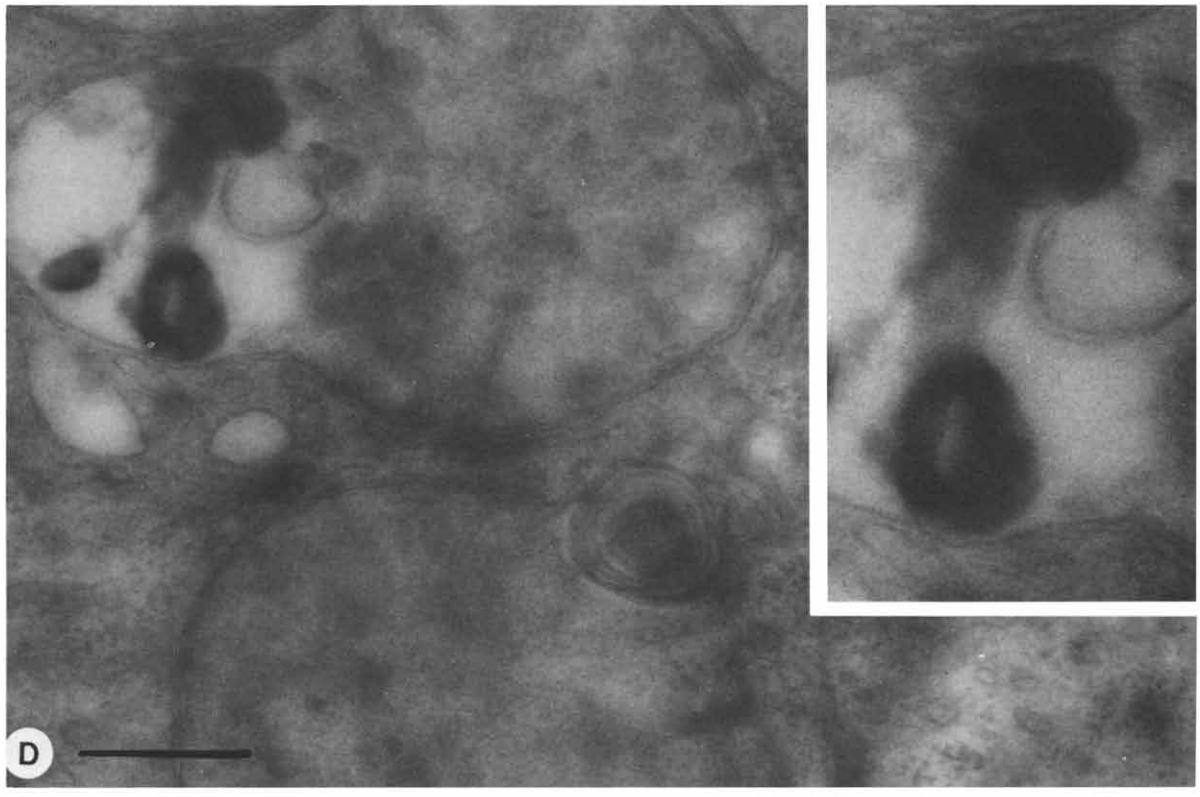
Liposomes were prepared at a concentration of 40 μmoles total lipid per ml. Thus 16 μmole PC, 4 μmole PS, and 20 μmole cholesterol were dissolved in chloroform. After removal of solvent, the dried lipids were taken up in ether. Uranyl acetate (UA, Polysciences Inc.) (UA/PL, 10 : 1 w/w), was sonicated in 0.5 ml phosphate-buffered saline (pH 7.4) and this was added to the lipid-ether solution. The resulting mix was vortexed, then the ether was removed under argon, breaking the gel several times by vortexing. The resulting liposomes were dialyzed overnight against phosphate-buffered saline. In some experiments, liposomes encapsulating buffer were prepared by the reverse phase evaporation method then incubated with uranyl acetate in phosphate-buffered saline (UA/PL, 10 : 1 w/w) at room temperature for 3–5 h. This was followed by overnight dialysis against phosphate-buffered saline. When non-encapsulated uranyl acetate was not completely removed during dialysis, intravenous injection resulted in immediate death of the animal.

When ferritin was encapsulated, liposomes were prepared as described above but feritin (Sigma) was added in phosphate-buffered saline, ferritin/PL (2 : 1, w/w). The sonication step was omitted. Non-encapsulated ferritin was removed by multiple washing and pelleting of liposomes.

Liposomes encapsulating uranyl acetate, buffer, or ferritin were uni- and oligo-lamellar, and ranged in size from about 1200 Å to 2500 Å.

Preparation of tissue for EM. Liposomes containing uranyl acetate or ferritin were injected in the tail vein of strain CD1 mice (Charles River), 4 μmoles total lipid per mouse. This corresponds to approximately $4 \cdot 10^{12}$ vesicles per mouse [11]. In some experiments, peroxidase (Worthington) was injected before the liposomes, 150 mg in phosphate-buffered saline per mouse. Liposomes were injected one hour later. Then at 20 min, 1 h or 4 h post-injection of the liposomes, the liver was excised and cut into 1 mm^3 pieces in the fixative solution at 4 °C. The fixative solution was 3% (w/v) glutaraldehyde (Polysciences Inc.) in 0.1 M sodium cacodylate/HCl buffer containing 0.03% $CaCl_2$ (pH 7.4). Tissue was then rinsed in several changes of buffer containing 0.3 M sucrose and 0.03% $CaCl_2$ (pH 7.4) and postfixed with 2% osmium tetroxide (Polysciences Inc.) buffered with cacodylate containing 0.15 M sucrose and 0.03% $CaCl_2$. This was followed by dehydration in a graded series of ethanol concentrations and inclusion in EPON (Polysciences Inc.). Tissue was kept at 4 °C through fixation procedures until dehydration, which was carried out at room temperature, as were all subsequent manipulations. Thin sections were cut using an LKB Ultratome IV, were mounted on copper grids and examined with a SIEMENS Elmiskop 102 electron microscope. In some cases, sections were poststained by soaking for 1 min in alkaline lead citrate (Polysciences

Fig. 1. (A–C) Thin sections of mouse liver. The animal was injected intravenously with liposomes encapsulating uranyl acetate. The liver was later excised and prepared for electron microscopy. The sections are not post-stained. (A) Association of endocytotic vacuole containing a liposome with a mitochondrion, 20 min post-injection. Magnificiation ×45000. Bar represents 0.33 μm. (B) Association of a liposome with a mitochondrion, 1 h post-injection. Liposome organization is clear. Outer and inner membranes and cristae of the mitochondrion are clear. Magnification ×80000. Bar represents 0.25 μm. (C) Association of an endocytotic vacuole containing liposomes with a mitochondrion, 1 h post-injection. Magnification ×45000. Bar represents 0.33 μm.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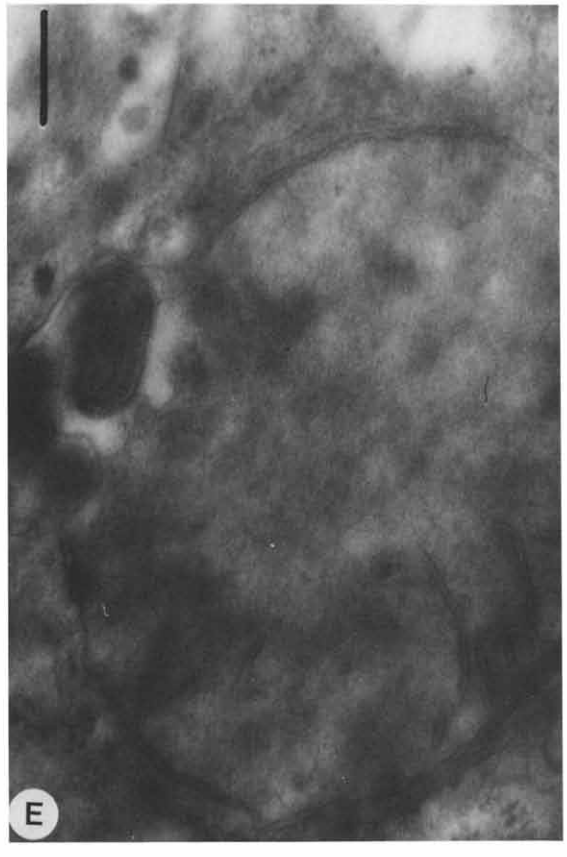
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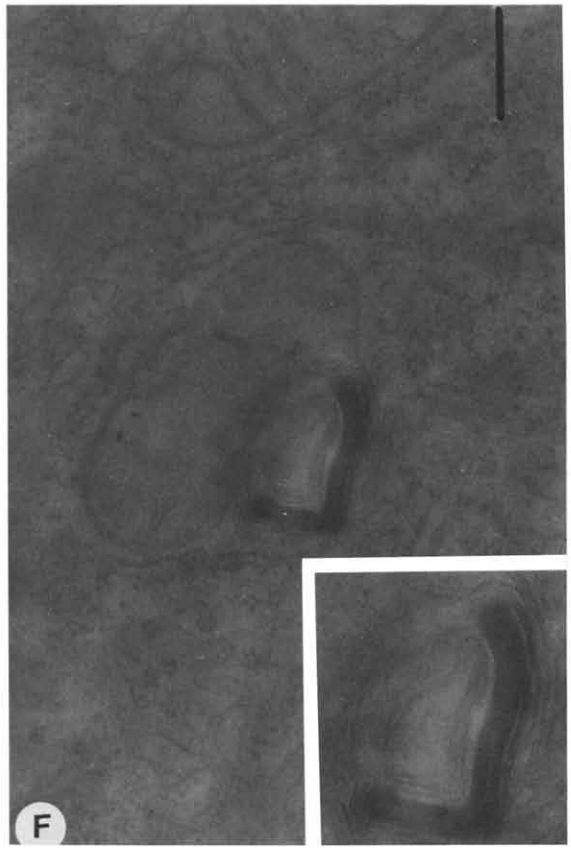
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Tissue used in freeze-fracture-etching analyses was cut into 1 mm^3 pieces in fixative solution at 4°C, immersed in fixative for 40 min, then transferred to a solution of 0.1 M cacodylate (pH 7.4), 4°C. Then a solution of 60% glycerol in cacodylate at pH 7.4 and 4°C was added with continuous swirling of the tissue until the final glycerol concentration was 30% (15% for etching). Tissue samples were placed in the recess of a 3-mm gold disc and then quenched at −160°C in Freon 22 cooled with liquid nitrogen. Specimen holders were fixed on the cooled stage of a Balzers 301 freeze etching unit. Fracturing was performed at −160°C (−100°C for etching) at a vacuum of about 10^{-6} Torr. Etching period was 60 s. The fracture surfaces were replicated with platinum (Pt) and carbon (C). Pt-C replicas were cleaned in solutions of bleach, collected on 300-mesh copper grids and examined in a SIEMENS Elmiskop 102 electron microscope.

Preparation of cells for EM. Mouse hepatocytes were recovered by perfusion with a collagenase-containing solution, as first described by Berry and Friend [12,13]. After low-speed centrifugation, the resulting pellet of hepatocytes was taken up in two or three drops of 2% agar (Difco) at 45°C. The cell suspension in agar was cooled on a glass slide, then cut into 1 mm^3 pieces and prepared for electron microscopy by the procedure used for tissue.

Results

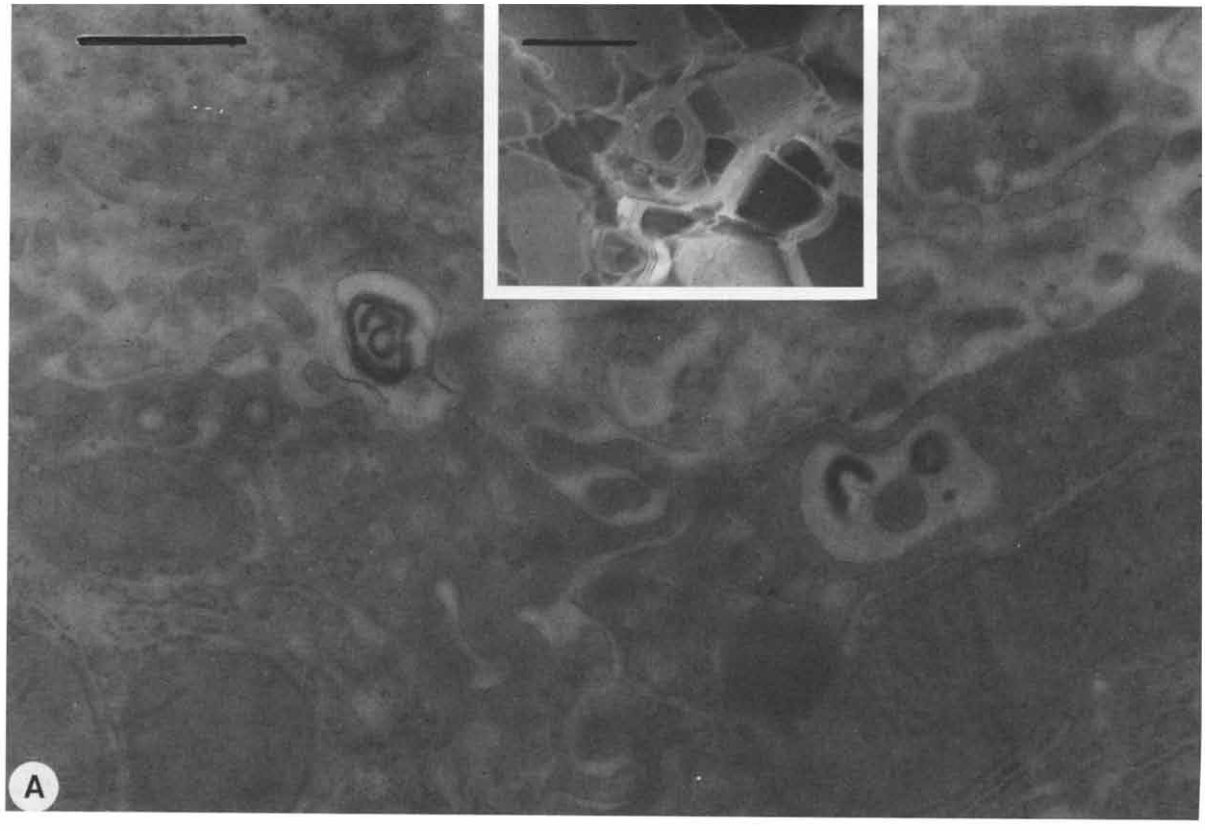
Livers were excised 20 min, 1 h and 4 h after injection of liposomes containing uranyl acetate. When visualized by transmission EM, the liposomes were darker than the surrounding liver tissue (they contained uranyl acetate) with the multiple repeating and regularly spaced lamellae of 40–60 Å characterizing oriented stacks of bilayers [14]. In most cases, the liposomes were clearly enclosed in a cytoplasmic vacuole. A significant association of vacuolated liposomes and mitochondria was observed. At one hour post-injection an average of one out of ten mitochondria was associated with a liposome. Analysis of significance using Student's *t*-test on 15 photographs indicated a 90–95% confidence level. Phagocytosis by Kupffer cells as well as fusion with primary lysosomes and inclusion in secondary lysosomes was also observed. Representative photographs are shown in Figs. 1 and 2.

Fig. 1A is a photograph taken from tissue excised 20 min after injection of PC/PS/Chol liposomes encapsulating uranyl acetate. The liposome is darker than surrounding tissue and is enclosed in a cytoplasmic vacuole. The subcellular organelle possesses the outer and inner membranes with folds of the inner membrane, the cristae, which define a mitochondrion. In this case the association appears to involve fusion of the cytoplasmic vacuole containing the liposome with the mitochondrial inner and/or outer membrane(s).

Fig. 1B was taken from tissue excised 1 h after injection. Here the organization of the liposome is clear, as is mitochondrial structure. Figs. 1C and 1D were taken from the same tissue. Several liposomes are in an endocytotic vacuole which appears to have fused with the mitochondrial membrane(s). Fig. 1E was taken 4 h after injection of liposomes containing uranyl acetate.

Lactosylceramide incorporated in the liposome bilayer has been shown to selectively enhance liposome uptake by endothelial cells and hepatocytes [13,15,16]. We wished to ascertain whether intracellular fate was altered as well. Thus, in some experiments lactosylceramide (LC) was included in the liposome bilayers (LC/PL, 1 : 10, mol/mol). Lactosylceramide was incorporated in the bilayers of the liposome shown in association with the mitochondrion in Fig. 1F. This tissue was excised 1 h post-injection. No difference was observed in intracellular fate when lactosylceramide was incorporated in the lipid bilayers of the liposomes.

Fig. 1. (D–F) Thin sections of mouse liver. (D) Association of an endocytotic vacuole containing liposomes with a mitochondrion, 1 h post-injection. The liposomes contain lactosylceramide in the bilayers. The inset shows an enlargement at the site of association. A continuous limiting membrane is evident, suggesting a fusion process in the association. Magnification ×67500. Bar represents 0.33 μm. (E) Four hours post-injection, liposomes may still be observed associating with mitochondria. Magnification ×60000. Bar represents 0.25 μm. (F) Association of a liposome with a mitochondrion, 1 h post-injection. The liposome contains lactosylceramide in the bilayers. Liposome organization is clear in the inset. Magnification ×45000. Bar represents 0.33 μm.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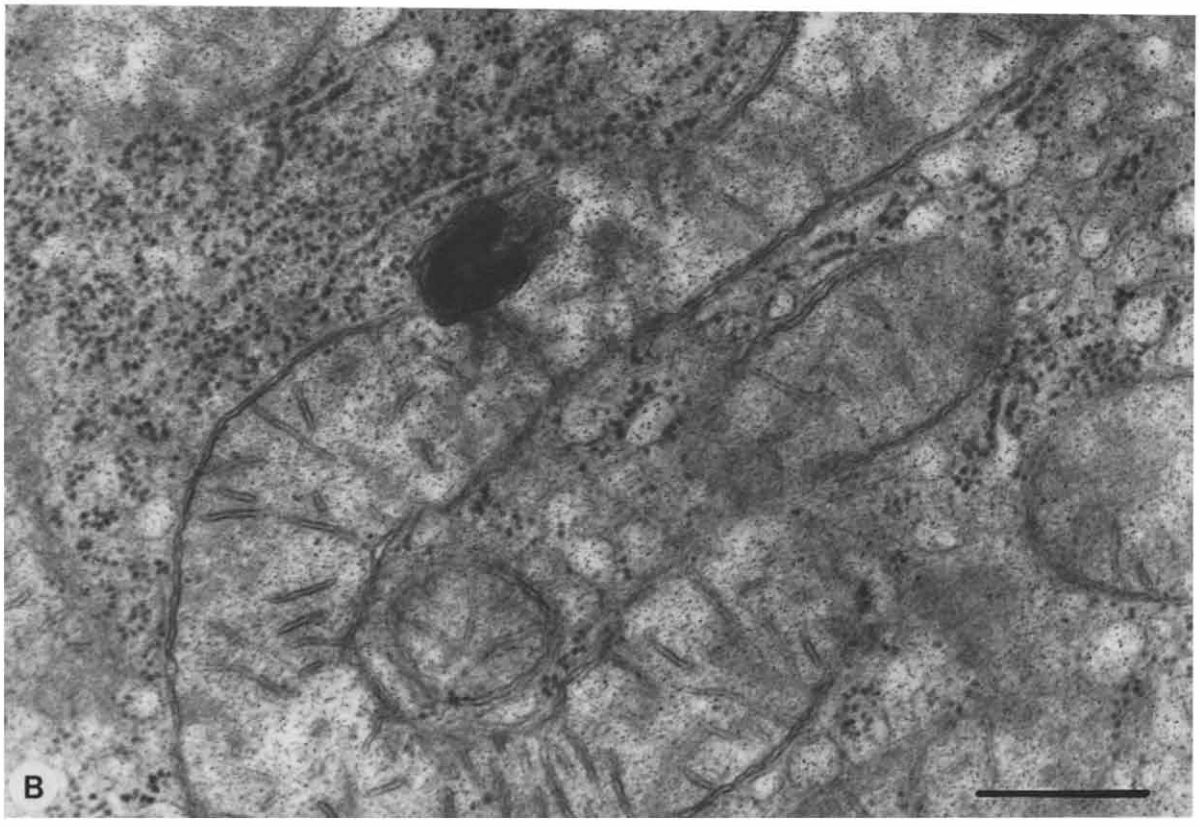
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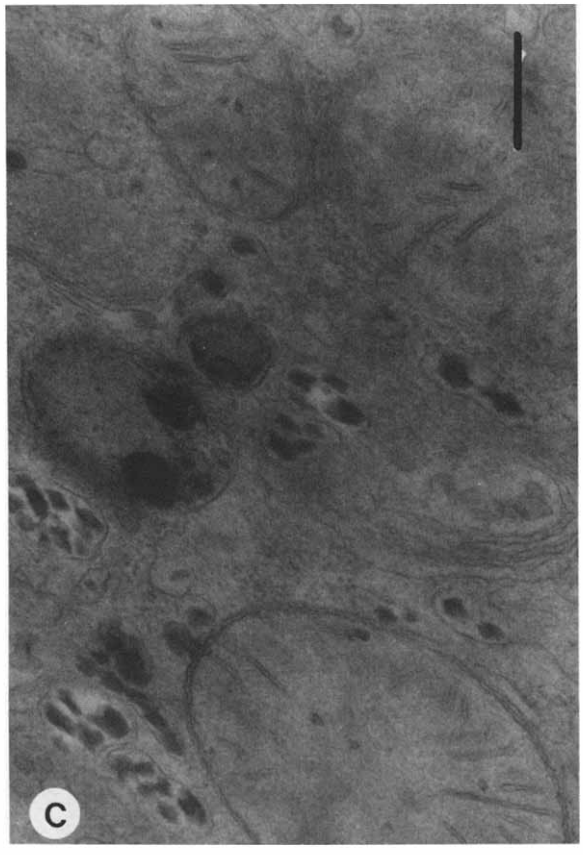


Fig. 2. Thin sections of mouse liver. The animal was injected intravenously with liposomes. The liver was later excised and prepared for electron microscopy. (A) These liposomes encapsulate buffer only. They were incubated in a solution of uranyl acetate in phosphate-buffered saline prior to injection. This tissue was excised 1 h post-injection. The liposomes are enclosed in endocytotic vacuoles and are found among endothelial microvilli and intracellularly. The section is not poststained. Magnification ×33750. Bar represents 0.67 μm. The inset shows liposomes prior to incubation with uranyl acetate. Magnification ×75000. Bar is 0.20 μm. (B) The same liposomes and tissue as (2A). Here the association of liposome with mitochondrion is shown. The section is post-stained with lead citrate. Magnification ×75000. Bar represents 0.40 μm. (C) Vacuoles filled with stained particles are seen here, 4 h post-injection. The dark particles may be lysosomal degradation products of liposomes, lipid assemblies containing uranyl acetate. The liposomes contained lactosylceramide in the bilayers. The section is not poststained. This phenomenon was commonly observed four hours post-injection. Magnification ×45000. Bar is 0.33 μm.

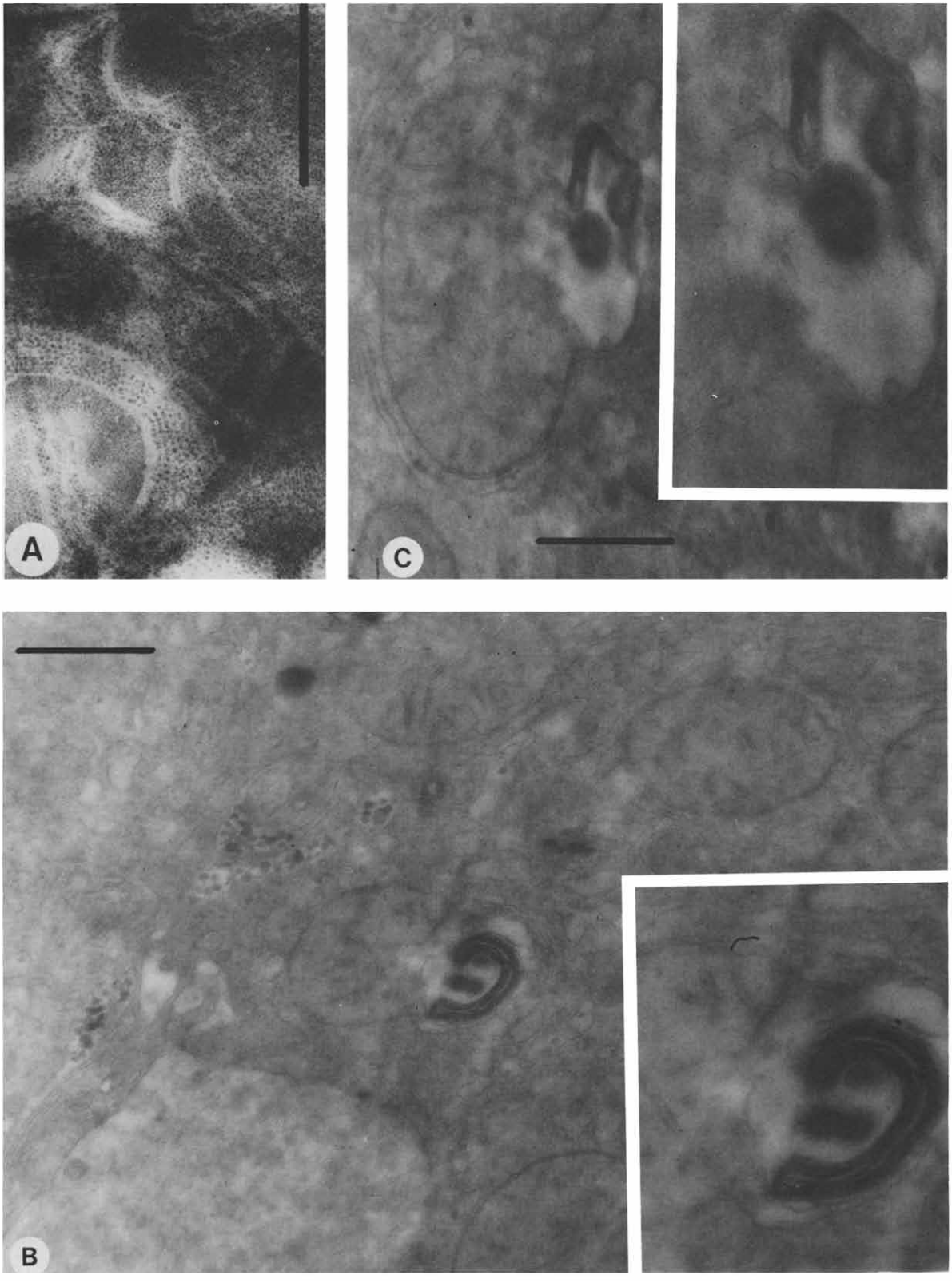
Liposomes were also observed as they were phagocytosed by Kupffer cells and associated with primary and secondary lysosomes (not shown).

Liposomes were prepared containing buffer. They were incubated with uranyl acetate, then dialyzed and injected intravenously. Representative photographs are shown in Figs. 2A and 2B. In Fig. 2A liposomes are seen in vacuoles among endothelial microvilli and intracellularly [17]. In Fig. 2B the association of liposome with mitochondrion is clear. Liposomes prior to incubation with uranyl acetate are shown in the inset in Fig. 2A.

In one experiment, horseradish peroxidase, a specific marker of lysosomes, 150 mg per mouse, was injected intravenously, then one hour later PC/PS/Chol liposomes were injected. One hour later the liver was excised. In another experiment, 150 mg per mouse was injected intravenously, then one hour later PC/PS/Chol/LC liposomes were injected. One hour later the liver was excised. In both cases, liposomes were found in cytoplasmic vacuoles in association with mitochondria. Liposomes were found, also, in association with lysosomes, marked in the experiments with peroxidase.

Fig. 2C was taken of tissue excised 4 h after liposome injection and shows the phenomenon which was frequently observed after 4 h but was not widespread after one hour only: this is the presence intracellularly and in the sinusoids of darkly stained particles in cytoplasmic vacuoles (the section is not poststained), believed to be assemblies of lysosomal degradation products of the uranyl acetate-loaded liposomes. It is reasonable to think that the phospholipase enzymes present in lysosomes would act on liposomes so as to disrupt their bilayer structures. Uranyl acetate might be expected to remain associated with the lysoPC/PC/cholesterol mixture, resulting in stained assemblies of lipid, as seen here.

Encapsulated ferritin has been used as a marker of liposomes under the electron microscope in studies in vitro [8,9]. We encapsulated ferritin in liposomes, injected intravenously, and examined liver tissue excised one hour after injection. Liposomes were visualized as darker than surrounding tissue (they contained ferritin) and in association with mitochondria. In most cases the liposomes were clearly enclosed in a cytoplasmic vacuole. Liposomes were also visualized in association with primary and secondary lysosomes. Representative photographs are shown in Figs. 3B and 3C. Lipo-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somes prior to injection are shown in Fig. 3A. In the photographs 3B and 3C, multilamellar liposomes enclosed in a cytoplasmic vacuole are in close association with a mitochondrion. Enlargement of the sites of association (insets) shows a continuous membrane structure where the mitochondrial membrane(s) join the vacuolar membrane. This suggests what might be seen as a fusion process in the association.

A freeze-fracture-etching analysis was carried out on liver tissue excised 20 min after injection of liposomes containing ferritin. Assemblies were found which could be interpreted as membrane-bound liposomes in association with mitochondria. These support earlier observations by transmission EM. Representative photographs are shown in Figs. 4A and 4B. Fig. 4A is a freeze etching which shows a cross-fractured mitochondrion. A cluster of vesicles associate with this mitochondrion (arrow). The vesicles are a size appropriate for liposomes (about 1800 Å diameter). Particles of a size about 120 Å may be seen on the etched faces of the vesicles. It is difficult to say with certainty whether these particles are ferritin or membrane proteins of a cytoplasmic vacuole. The appearance of the fracture is suggestive of a fusion process between what may be interpreted as liposomes or cytoplasmic vacuole and a mitochondrial membane(s). Fig. 4B is a freeze-fracture and shows the outer face of an external mitochondrial membrane over the outer face of its internal membrane. Several vesicles appose the mitochondrion. The vesicles have diameters of about 1400 Å. A few membrane particles are seen on the surfaces of convex vesicles. This is to be expected if they are liposomes enclosed in a cytoplasmic vacuole(s). No membrane particles are seen on the inner surface of the concave vesicle in Fig. 4B (arrow), a strong indication that this is a liposome. However, close observation at the circumference of this vesicle reveals particles on the surface.

From morphological characteristics such as disposition with respect to sinusoids, Kupffer cells, endothelial microvilli as well as the shape of the cell nucleus, the mitochondria which associate with liposomes appear to be primarily those of hepatocytes [17]. When liposomes containing uranyl acetate were intravenously injected and hepatocytes were isolated by collagenase perfusion one hour later, transmission EM revealed the presence of liposomes in these cells, in cytoplasmic vacuoles in the cytoplasm and in association with mitochondria. Representative photographs are shown in Figs. 5A and 5B. Fig. 5A shows liposomes containing uranyl acetate in cytoplasmic vacuoles in the cytoplasm in isolated hepatocytes. In Fig. 5B, the association with a mitochondrion is seen. However, on the basis of electron microscopy only, we cannot rule out liposome association with mitochondria of endothelial cells or with Kupffer cell organelles. Liposomes were observed being phagocytosed by Kupffer cells (not shown) and frequently among endothelial microvilli (Fig. 2A).

In a control experiment, normal rat liver was excised and processed for electron microscopy in the same way as were livers of mice injected with liposomes. Microscope examination of normal rat liver, after uranyl acetate post-stain of the thin sections, revealed the presence of myelin bodies. We believe we do not confuse these structures with liposomes for three reasons. First: the myelin structures are not strongly contrasted to the rest of the tissue in unstained sections, as liposomes are since they encapsulated uranyl acetate or ferritin. Second: the myelin structures are significantly larger than liposomes, myelin structures averaging 0.77 μm in diameter and liposomes averaging 0.20 μm. Third: lamellae of myelin structures are quite irregularly spaced while lamellae of our liposomes have the expected regular repeat distance.

Fig. 3. (A) Liposomes encapsulating ferritin, prior to injection. The liposomes are negatively stained with ammonium molybdate (pH 7.0). Multilamellae of the liposomes are defined by encapsulated ferritin. Magnification ×120000. Bar represents 0.25 μm. (B) Thin section of mouse liver. The animal was injected intravenously with liposomes encapsulating ferritin. The liver was excised one hour later and prepared for electron microscopy. The section is not post-stained. The association of liposome with mitochondrion is apparent. Liposome organization is clear in the inset. Magnification ×33750. Bar represents 0.67 μm. (C) Same tissue as (3B). Association of liposome with mitochondrion is shown. In the inset the continuity of a limiting membrane at the site of association is clear. Magnification ×45000. Bar is 0.50 μm.

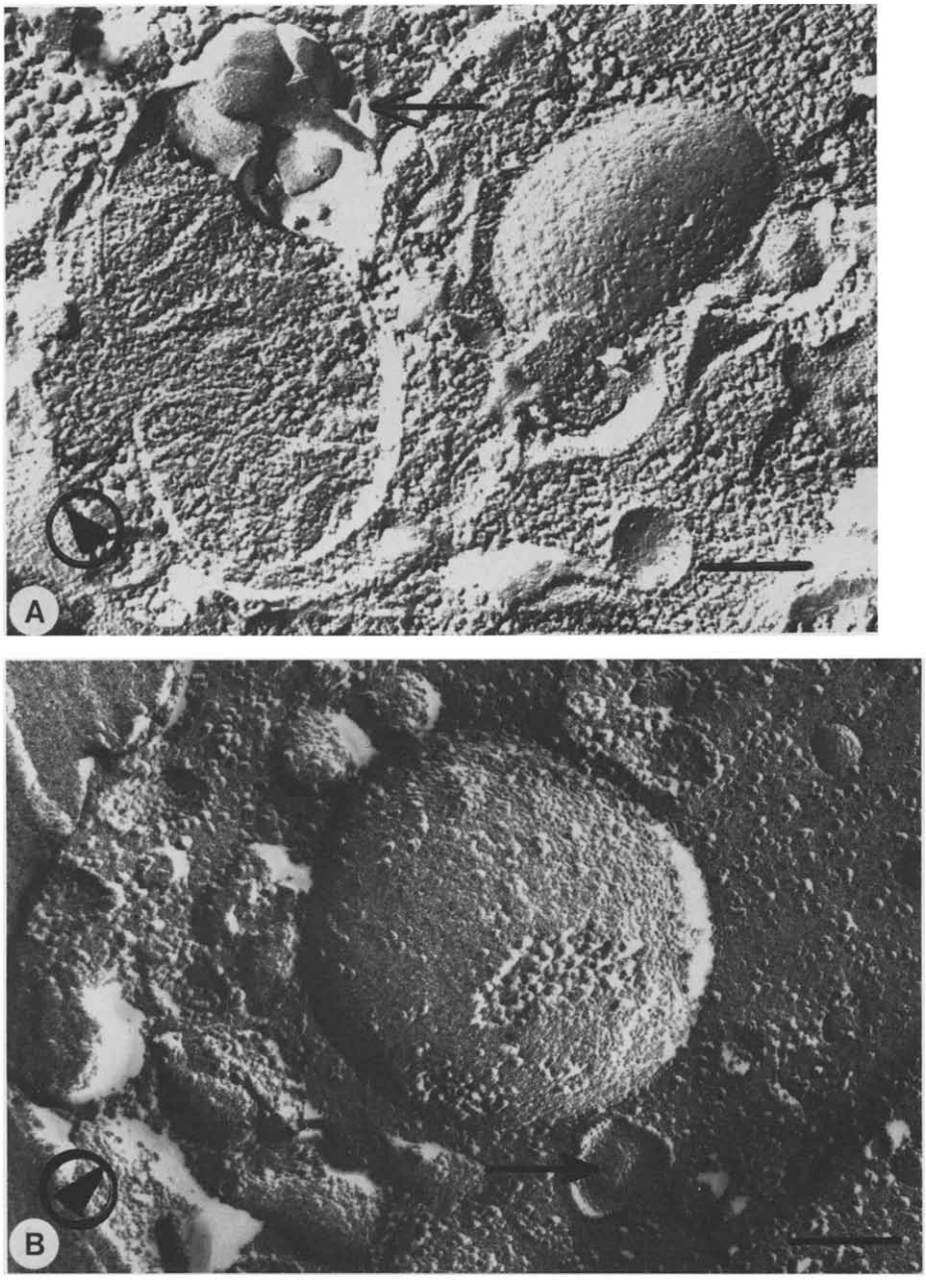
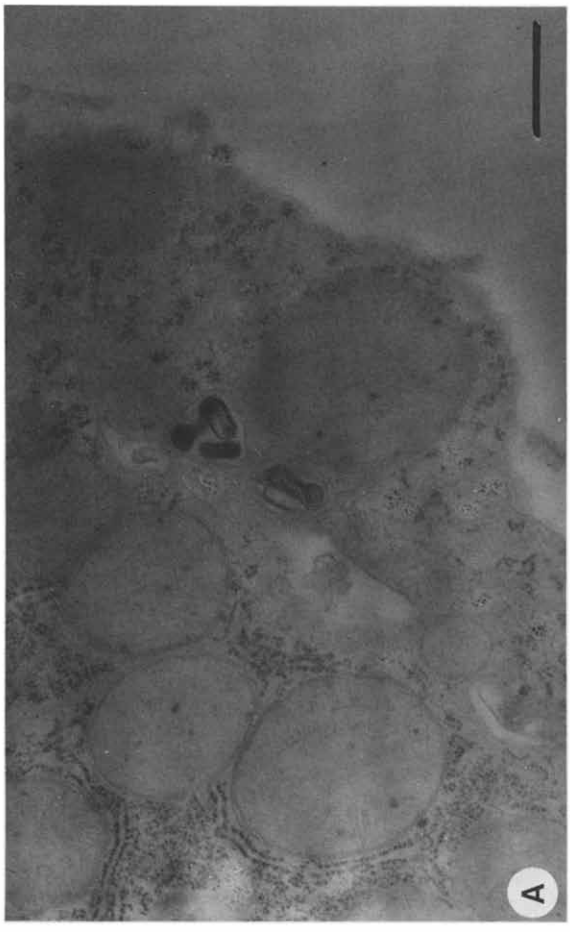


Fig. 4. Freeze-fracture-etching images of mouse liver excised 20 min after injection of liposomes containing ferritin. (A) A freeze-etching showing a cross-fractured mitochondrion associated with a cluster of vesicles. Particles of a size appropriate for ferritin (about 120 Å) are seen on the etched faces of the vesicles, but it is difficult to say with certainty that these particles are not intramembrane proteins of a cytoplasmic vacuole around the vesicles. Magnification ×61 875. Bar is 0.24 μm. (B) A freeze-fracture of the same experiment on another animal. Vesicles (1400 Å diameter) appose this mitochondrion. Membrane particles are seen on the convex surfaces. No membrane particles appear on the inner surface of the concave vesicle (arrow), a strong indication that this is a liposome. Close observation at the circumference of this vesicle reveals particles of the surface. Magnification ×80 000. Bar represents 0.17 μm.



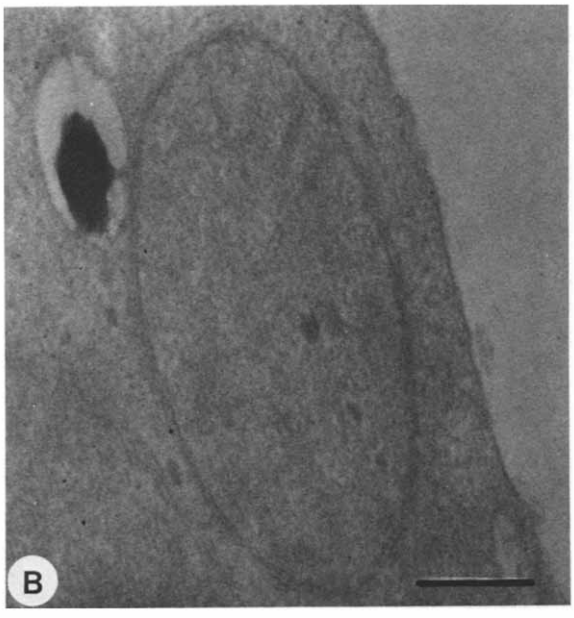


Fig. 5. Mouse hepatocytes, recovered by collagenase perfusion one hour after injection of liposomes encapsulating uranyl acetate. The sections are not post-stained. (A) Liposomes enclosed in the endocytotic vacuoles in the cytoplasm of recovered hepatocytes. Magnification in (A) ×22500. Bar represents 0.67 μm. (B) Association of endocytotic vacuole containing liposome with a mitochondrion. Magnification in (B) ×75000. Bar is 0.20 μm.

Discussion

Multiple sections from livers of fourteen animals were examined: normal rat; mouse injected with horseradish peroxidase only; mouse with PC/PS/Chol liposomes containing uranyl acetate only, 20 min, 1 h, 4 h post-injection; mouse with PC/PS/Chol/LC liposomes containing uranyl acetate only, 1 h, 4 h post-injection; mouse with peroxidase then PC/PS/Chol liposomes containing uranyl acetate, 1 h post-injection; mouse with peroxidase then PC/PS/Chol/LC liposomes containing uranyl acetate, 1 h post-injection; mouse with PC/PS/Chol liposomes containing ferritin, three experiments at 20 min and one at 1 h post-injection; and hepatocytes from mouse liver, recovered by collagenase perfusion 1 h post-injection of PC/PS/Chol liposomes containing uranyl acetate. Results from all experiments were identical: in all cases where liposomes were injected, they were found in cytoplasmic vacuoles in association with mitochondria. Liposomes were also found, as expected, associated with primary and secondary lysosomes and among endothelial microvilli. Vacuolated liposomes were found in association with mitochondria in hepatocytes recovered by collagenase perfusion. Freeze-fracture analysis supported findings by transmission EM.

It is tempting to speculate that liposomes in vivo interact with subcellular organelles randomly with a statistical frequency. Since liver cells are replete with mitochondria (1000–1600 per cell), liposomes would be observed associating with them more often than with other, less numerous, organelles. In these experiments, photographs taken at low magnification show approximately one mitochondrion out of ten in association with liposomes at one hour post-injection. Analysis of

significance using Student's *t*-test on 15 photographs indicated a 90–95% confidence level. If the association is a statistical phenomenon, however, this ratio will change for sections from other parts of the liver. The liposome distribution in the liver may not be uniform after one hour and further, the number of mitochondria per cell is not uniform in the liver (peripheral cells: 1060; central cells: 1600) [18].

Association of mitochondria with lipid assemblies has previously been reported. Thus it has recently been shown that *Legionella pneumophila*, the agent of Legionaires' disease, resides in human monocytes in a membrane-bound cytoplasmic vacuole which becomes surrounded by mitochondria closely apposed to the vacuolar membrane [19]. Further, it is known that mitochondria are found in close relation to lipid droplets in the cytoplasm of the pancreatic acinar cell, where the lipid droplet may serve as a supply of substrate for the mitochondrion, particularly after the animal is fasted [20].

The electron micrographs shown here demonstrate that endocytotic vacuoles containing liposomes associate with mitochondrial membrane(s) and ultimately, one would think, enter the mitochondrial inner chamber. Presumably, liposomal contents are discharged into the mitochondrial matrix. The biochemical significance of this event remains to be determined.

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