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Interaction of intravenously injected liposomes with mouse liver mitochondria. A fluorescence and electron microscopy study

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Megamitochondria, resulting from cuprizone feeding of Swiss ICR mice, were fluorescent in hepatocytes after the intravenous injection to mice of a liposome-encapsulated acridine orange-DNA complex (AO-DNA). Flow cytofluorimetric analysis of isolated megamitochondria showed that the proportion of liposome-encapsulated AO-DNA which localized in megamitochondria increased from 0.02% of the dose injected per liver cell at 3 min after injection to an average of 0.34% at 1 h after injection. Megamitochondria showed negligible fluorescence by fluorescence activated cell sorting (FACS) analysis when free AO-DNA was intravenously injected. Transmission electron micrographs of mouse liver tissue after intravenous injection of liposomes encapsulating iron dextran showed an association of the liposomes with megamitochondria which appeared identical to liposome association with normal mitochondria. These results support and extend our earlier observation that a fraction of the liposomes injected intravenously into mice associate with mitochondria in the liver, and possibly deliver their aqueous contents there.

Introduction

The possibility that liposomes transfer their contents to liver mitochondria after intravenous injection to mice has been raised by previous electron microscope studies from this laboratory [1,2].

Transmission electron microscopy aimed at visualizing the intracellular fate of liposomes and their contents showed that liposomes injected intravenously into mice associate with mitochondria in the liver [1]. Subsequent studies of the intracellular fate of intravenously injected lipsome-encapsulated DNA in mouse liver using electron microscope autoradiography and subcellular fractionation have supported this finding [2]. In the latter study, EM autoradiography showed that the proportion of DNA associated with mitochondria steadily increased for one hour after injection. Part of the DNA followed the subcellular fractionation profile of the mitochondrial matrix marker, malate dehydrogenase. We here present further evidence using fluorescence markers analysis supported by electron microscope studies which shows transfer of encapsulated material by liposomes to liver mitochondria. Normal liver mitochondria being too small for light-microscope examination, we resorted to the use of mice megamitochondria. Megamitochondria are readily induced in mouse liver by administration of cuprizone (bis-cyclohexanone oxaldihydrazone) in the

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol; LC, lactosylceramide; AO, Acridine orange; FACS, fluorescence activated cell sorting; EM, electron microscopy.

animal diet for more than 4 days [3–7]. Such mitochondria, having diameters of 0.5–7.0 μm, can be visualized by light microscopy in tissue [5] or in suspension after cell disruption and isolation by centrifugation [7]. The presence of a fluorescent dye associated with a megamitochondrion may be observed [8,9]. Ultrastructural details of megamitochondria in tissue and of isolated megamitochondria have been documented using transmission EM [3–6].

We here report that megamitochondria are fluorescent in hepatocytes isolated one hour after intravenous injection of liposome-encapsulated acridine orange-DNA complex, as observed by fluorescence microscopy. In order to quantitate this observation, we have used flow cytofluorimetry (FACS) analysis to estimate amounts of fluorescence associated with megamitochondria isolated from mouse liver at 3 min, 30 min, and 1 h after injection. FACS analysis showed that the amount of dye associated with megamitochondria increases over 1 h for the case of liposome-encapsulated AO-DNA. Negligible amounts of dye are associated with megamitochondria 1 h after free AO-DNA is intravenously injected. Observations from fluorescence experiments are supported by transmission electron micrographs showing interaction of megamitochondria with liposomes which encapsulate iron dextran.

Materials and Methods

1. Megamitochondria in mice

Cuprizone (bis-cyclohexanone oxaldihydrazone) (Sigma, F.R.G.) was purified by recrystallization from ethanol [9]. The purified crystals were stored at −20°C. Cuprizone was administered in standard laboratory rodent chow [9]. Thus, about one week prior to use, cuprizone was added to ground rodent chow in a Waring blendor, 3 g cuprizone per 500 g chow, i.e., 0.6% cuprizone. The resulting mixture was stored in the dark at 4°C until use. Unweaned Swiss ICR mice (Centre de Sélection et d'Elevage d'Animaux de Laboratoire, CNRS, Orléans), 17 days old of either sex, were administered the cuprizone-chow mix and supplied with deionized water. Mice were kept in plastic cages and used for experimentation after 7 days and up to 4 weeks after initiation of the diet of cuprizone. Mice were supplied with the cuprizone diet after liposome injection and up to the moment of death, so that mitochondrial normalization would not occur [4,5].

2. Liposomes encapsulating acridine orange-DNA

Acridine orange and deoxyribonucleic acid from Coliphage T7 were used as purchased from Sigma. Acridine orange, 10^{-3} M, in 0.001 M sodium acetate (pH = 6.75) was added dropwise with shaking to a solution of DNA in 0.001 M sodium acetate, at room temperature. Acridine orange was added in quantity which was 1 : 1, mole : mole, with DNA phosphate, using an ε of 6550 for DNA absorbance at 259 nm [10]. The resulting complex was shielded from direct light. After about 30 min at room temperature, the AO-DNA complex was isolated by routine precipitation, i.e., to 0.500 ml of AO-DNA solution in Eppendorf tubes, 5 M NaCl was added to a final concentration of 0.15 M NaCl. Two volumes of ethanol were added, and the solution was placed at −70°C for 30 min. After table-top centrifugation, the supernatant was decanted and residual liquid was evaporated under vacuum. The resulting pellet was taken up in 0.001 M sodium acetate (pH = 6.75) and used for encapsulation in liposomes. Fluorescence spectra were obtained using a Fica MK-II double-beam spectrofluorometer or a Perkin-Elmer LS 5 spectrofluorometer. The wavelength of excitation was 490 nm and the wavelength of emission was 530 nm. Quantitative calculations involving the AO-DNA complex were made using the molecular weight of the uncomplexed plasmid ($25 \cdot 10^6$). Absorption and fluorescence spectra of AO-DNA in solution in sodium acetate did not change over a period of five days when the AO-DNA complex was kept at 4°C in the absence of light.

Liposomes were comprised of PC/PS/Chol/LC in the mole ratio, 4 : 1 : 5 : 0.5. Egg yolk phosphatidylcholine (PC) and cholesterol (Chol) were from Sigma, F.R.G.; phosphatidylserine (PS) was from Lipid Products, U.K., and *N*-stearoyl-DL-dihydrolactocerebroside (LC) was from Bio-Yeda, Israel.

Liposomes were prepared by reverse phase evaporation [11] as modified by Nicolau et al. [12] at a concentration of 40 μmol phospholipid/ml.

Thus, for 0.250 ml of liposomes, 8 μmol PC, 2 μmol PS, 10 μmol Chol and 1 μmol LC were evaporated in 0.5 ml freshly distilled chloroform. The lipids were taken up in 0.750 ml of peroxide free diethyl ether. Then 0.250 ml of a solution of AO-DNA in sodium acetate was added. The resulting solution was mixed by vortexing, and ether was slowly removed by rotary evaporation under nitrogen. The gel which formed was broken by vortexing. The resulting suspension of liposomes was washed with 4 ml of phosphate-buffered saline (pH 7.2) and concentrated by centrifugation at $115\,000 \times g$ for 30 min, two times. The resulting pellet was treated with DNAase I (Boehringer-Mannheim, France) in 10 mM $MgCl_2$ in phosphate-buffered saline at 37°C for 10 min. The suspension was then filtered through a Sepharose 4B column using phosphate-buffered saline. Acridine orange fluorescence emission at 530 nm was monitored. Liposomes eluted in the void volume and were concentrated by centrifugation at $115\,000 \times g$ for 30 min. Normalization of fluorescence emission intensities of the AO-DNA solution prior to encapsulation and of the final liposome suspension to solutions of known dye concentration indicated an encapsulation of about 25%. In experiments at 3 and 60 min, the DNA concentration was 10^{-8} M (0.025 mg per 100 μl), and the dye concentration was $2.5 \cdot 10^{-5}$ M. In the experiment at 30 min, the DNA concentration was $6 \cdot 10^{-8}$ M, and the dye concentration was $1.5 \cdot 10^{-4}$ M. This corresponds to a DNA : AO ratio of $4 \cdot 10^{-4} : 1$ in both cases. At 10^{12} liposomes per μmol of total lipid [13], one DNA molecule was encapsulated per 12 liposomes. Liposomes were stored under argon in phosphate-buffered saline at 4°C and used within 3 days.

3. Fluorescence analysis

Liposomes encapsulating AO-DNA were injected in the tail vein of Swiss ICR mice which had been on a diet of cuprizone for at least seven days. Mice weighed between 8 and 12 g. 8–16 μmol total lipid was injected per mouse in 100–200 μl of liposome suspension. Mice were maintained on a diet of cuprizone and bidistilled water after injection until the moment they were sacrificed, to prevent normalization of megamitochondria. The animals on the cuprizone diet were smaller, were less active, weaker, and more fragile than normal animals, in agreement with other reports [5,6]. Liver perfusions were difficult to carry to completion successfully, generally requiring more than one attempt in an experiment. Thus it resulted that animals from a range of times on the cuprizone diet were used.

Hepatocytes were recovered using a slight modification of the method of Berry and Friend [14,15]. That is, animals were anesthetized with diethyl ether, livers were perfused with collagenase-containing solution, and hepatocytes were recovered after gentle centrifugation. Isolated hepatocytes were diluted with a Krebs-Ringer-Hepes solution and stored on ice. Cell viability was 60–80% by the criterion of Trypan blue exclusion. Aliquots of whole cell suspensions in Krebs-Ringer-Hepes solution were placed on glass slides, protected with cover slips and viewed at room temperature using a Zeiss Universal microscope equipped with a standard filter for green excitation (Zeiss no. 487715). Fluorescence analysis was carried out immediately and continued without interruption for periods up to 4 h after isolation of the cells. Significant shrinkage of mitochondria was not detected.

Megamitochondria for the FACS analysis were isolated according to the general procedure outlined by Bowman and Tedeschi [9]. Thus, at times 3 min, 30 min, and one hour (two experiments) after injection, the animal was anesthetized with diethyl ether, and the liver was perfused with 5–10 ml of 0.30 M sucrose (pH 7.0). After homogenization of the tissue, megamitochondria were isolated by centrifugation in sucrose solutions as described [9]. Preparations were checked for mitochondrial integrity and for contamination by other organelles using transmission EM. Thus, aliquots from suspensions of isolated megamitochondria were placed on collodion-covered grids, stained for 30 s with a solution of ammonium molybdate (pH 7.4) and examined using a Siemens Elmiskop 102 electron microscope. Preparations of megamitochondria were stored in sucrose-Hepes solution on ice until flow cytofluorimetric (FACS) analysis. Suspensions of megamitochondria were diluted with sheath fluid immediately before FACS analysis. No significant shrinkage of mitochondria in this solution in the time required for analysis was

detected by light microscope examination. The FACS analyses were carried out within 2 h of isolation of mitochondria.

Fluorescence intensities and sizes of megamitochondria were estimated using a FACS analyser (Becton-Dickinson), λ (excitation): 485 nm, bandwidth: 20 nm, and λ (emission): 530 nm, bandwidth: 30 nm. The analyser was standardized using polystyrene sulfonate beads, 3 ± 0.1 μm and 6 ± 0.1 μm containing 1-(fluoresceinylthioureido)-4,8-diaza-eicosane [16]. To estimate the molar concentrations of dye in megamitochondria, the relative fluorescence values obtained from the FACS analysis were normalized to a solution of AO-DNA of known molarity by spectrofluorimetric comparison of the AO-DNA solution ($\lambda_{ex} = 490$ nm; $\lambda_{em} = 530$ nm) with a suspension of fluorescent beads which was later used in the FACS analysis ($\lambda_{ex} = 495$ nm; $\lambda_{em} = 525$ nm).

In each FACS analysis (each experiment), 23000 to 50000 particles were counted. The analyser was adjusted to recognize only particles having diameters greater than about 1 μm, to avoid counting and analysis of small vesicle contamination.

4. Liposomes encapsulating iron-dextran

Iron dextran was obtained in solution, 200 mg iron/ml (as a gift from Sanofi Santé Animale, St.-Jean-de-la-Ruelle, France). 100 ml of a 1:10 dilution with phosphate-buffered saline could be injected intravenously into a mouse having megamitochondria, without apparent toxicity.

Reverse phase liposomes comprised of PC, PS, and Chol (4:1:5, mole:mole) were prepared as described previously [1], using undiluted iron dextran. Unencapsulated iron dextran was separated from liposomes using a Sephadex G-25 column having dimensions 0.5 cm by 10 cm. The eluted liposomes were concentrated by centrifugation. Liposomes were stored under argon in phosphate buffered saline at 4°C and used within one week.

5. Electron microscopy

Liposomes encapsulating iron dextran were injected in the tail vein of Swiss ICR mice which had been maintained on a diet of cuprizone for at least seven days. 4 μmol total lipid were injected per mouse, corresponding to about $4 \cdot 10^{12}$ liposomes [13]. 10 min after injection, the animal was anesthetized with diethyl ether, the liver was perfused with 5–10 ml phosphate-buffered saline, excised, and prepared for transmission EM as described earlier [1]. Thin sections were examined without poststain using a Siemens Elmiskop 102 electron microscope.

6. Control experiments

Hepatocytes from mice which had been administered cuprizone but had not been injected were examined by fluorescence microscopy for autofluorescence of whole cells or megamitochondria. In another experiment, 0.150 mg free AO-DNA was injected intravenously. One hour later the hepatocytes were isolated and examined by fluorescence microscopy for cytoplasmic fluorescence or fluorescence of megamitochondria.

So far as control for the FACS analysis of megamitochondria is concerned, the absence of fluorescing megamitochondria after intravenous injection of 0.150 mg of free AO-DNA, indicated that megamitochondria do not exhibit significant autofluorescence at excitation and emission wavelengths of interest.

For electron microscopy, the control experiment consisted in injection of 100 μl of 'Gleptosil' which had been diluted 1:10 with phosphate-buffered saline, i.e. 2 μg of free iron dextran, followed by excision and preparation of the liver tissue for electron microscopy one hour later, using the same methods as were employed for the experiment with liposome-encapsulated iron dextran. The liver from a mouse which had been administered cuprizone but had not received any injection was also prepared for transmission EM and examined.

Results and Discussion

1. Intercalation of Acridine orange into bacteriophage T7-DNA and encapsulation in liposomes

Intercalation of Acridine orange into bacteriophage T7-DNA resulted in a slight shift of the emission wavelength maximum of the dye, from 536 nm for free acridine orange to 533 nm for intercalated dye ($\lambda_{ex} = 490$ nm). The position of the maximum indicates that Acridine orange has intercalated into native, as opposed to denatured,

DNA [17]. However, the spectrum from the solution of intercalated dye may represent a mixture of fluorescing species. Fluorophores which may be present include free Acridine orange, possibly monomer and dimer, in equilibrium with intercalated dye, dye which is complexed with denatured segments of DNA, and Acridine orange intercalated into native DNA [17–19]. Thus an exact formulation of the fluorescing species may not be made.

Encapsulation of this material into liposomes resulted in a suspension of liposomes fluorescing in a broad band with a wavelength maximum of about 530 nm when excited at 490 nm. The liposomes contained a bright green fluorescence against a dark background under the fluorescence microscope using a fluorescein filter. Liposome average size was 0.25 μm, from measurements of negatively stained liposomes in electron micrographs.

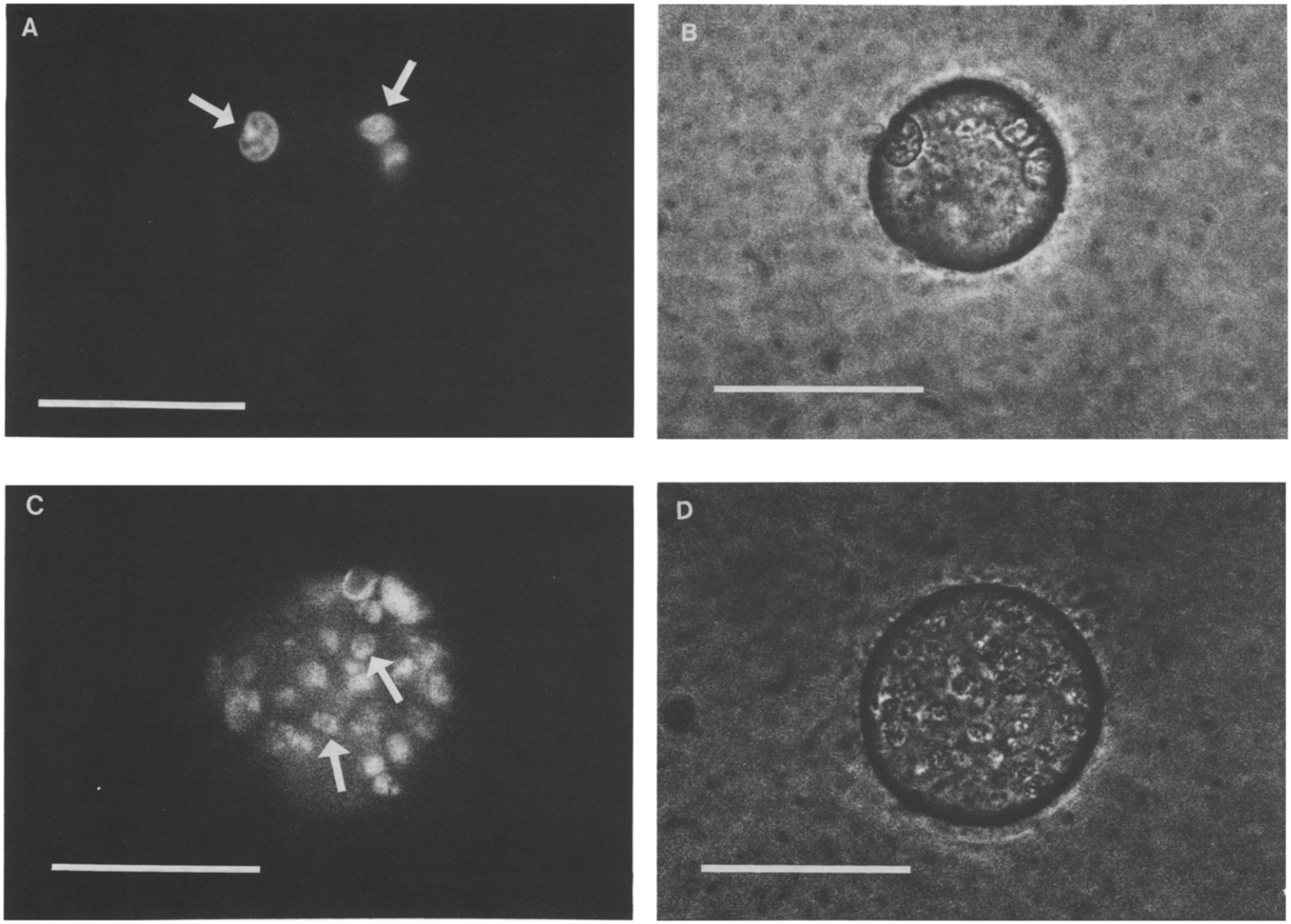
2. Fluorescence microscopy of hepatocytes

Some hepatocytes contained areas of concentrated fluorescence having a size, shape, and internal configuration appropriate for megamitochondria (Figs. 1A–H and Table I). In Fig 1A, for example, the fluorescing areas have sizes 2.2 μm $\times$ 1.7 μm, 2.1 μm $\times$ 1.7 μm, and 1.7 μm $\times$ 1.6 μm. In our hands, megamitochondria ranged in size from 0.5 μm to 6.7 μm (Table I, all methods of visualization). Normal rat liver mitochondria range in size from 0.5 μm to 1.1 μm [20]. Fig. 1A shows that Acridine orange dye was not spread uniformly in the mitochondria but appeared to be concentrated between the inner and outer membranes. What may be interpreted as cristae, invaginations of the inner membrane, appeared to be filled with dye.

Figs. 1C and 1D show another hepatocyte, larger, and having numerous areas of concentrated fluorescence. The cell shown here has a diameter of about 12 μm. Areas of fluorescence have diameters between 0.9 μm and 1.7 μm, thus they are a size appropriate for slightly enlarged mitochondria, not liposomes or other organelles. (The mean diameter of a rat parenchymal cell nucleus is 8.1 ± 1 μm and peroxisome average diameter is 0.64 ± 0.02 μm [20]. Average liposome diameter was 0.25 μm.) Some compartmentalization of fluorescence may be seen in the mitochondria (arrows, Fig. 1C). The cytoplasm in this cell is weakly fluorescent; however, nothing can be seen which could be interpreted as a fluorescent nucleus. Such an absence of fluorescent nuclei in our experiments, even as long as one hour after intravenous injection, indicated that dye did not become free in the cell. For example, it has been shown that cells which are supravitally stained with Acridine orange accumulate dye in the lysosomes ($\lambda_{ex} = 380$ nm; $\lambda_{em} = 650$ nm) and in the nucleus, where the dye interacts with single-stranded DNA ($\lambda_{ex} = 380$ nm; $\lambda_{em} = 640$ nm), with double-stranded DNA ($\lambda_{ex} = 380$ nm; $\lambda_{em} = 530$ nm) and with RNA [21]. This is the case for different kinds of cells and for cells which have been permeabilized and those which have not [22,23]. Thus we interpreted lack of fluorescent nuclei as evidence that liposomes in the cell did not release their contents indiscriminately into the cytoplasm and that the liposome-encapsulated AO-DNA complex which was injected intravenously remained intact, at least until after it was delivered intracellularly.

Fluorescent mitochondria having diameters of about 3.5 μm are shown in Fig. 1E. Contours of the adjacent non-fluorescent nucleus can be seen in Fig. 1F. In Figs. 1G and H, only two cells (center) contain fluorescent mitochondria. The diameter of the largest fluorescent mitochondrion is 2.3 μm. Fluorescence photographs, taken prior to phase contrast, required exposure times of about three minutes and led to frequent cell mortality.

Not every hepatocyte contained fluorescent areas. The percentage of cells with fluorescence could not be reliably estimated by microscope examination due to technical problems, some of which have been reported by others [24]. For example, hepatocytes were fragile under the illuminating light and frequently died during the long exposure times required for recording fluorescence; when fluorescence in an organelle was concentrated, for example, in small vacuoles, the vacuoles often burst under illuminating light, releasing fluorophore into the cytoplasm. Fluorescence faded over the exposure time required for photography. And, over the four hour examination time, even cells kept at 0–4°C showed some changes in patterns of fluorescence.


A
B
C
D

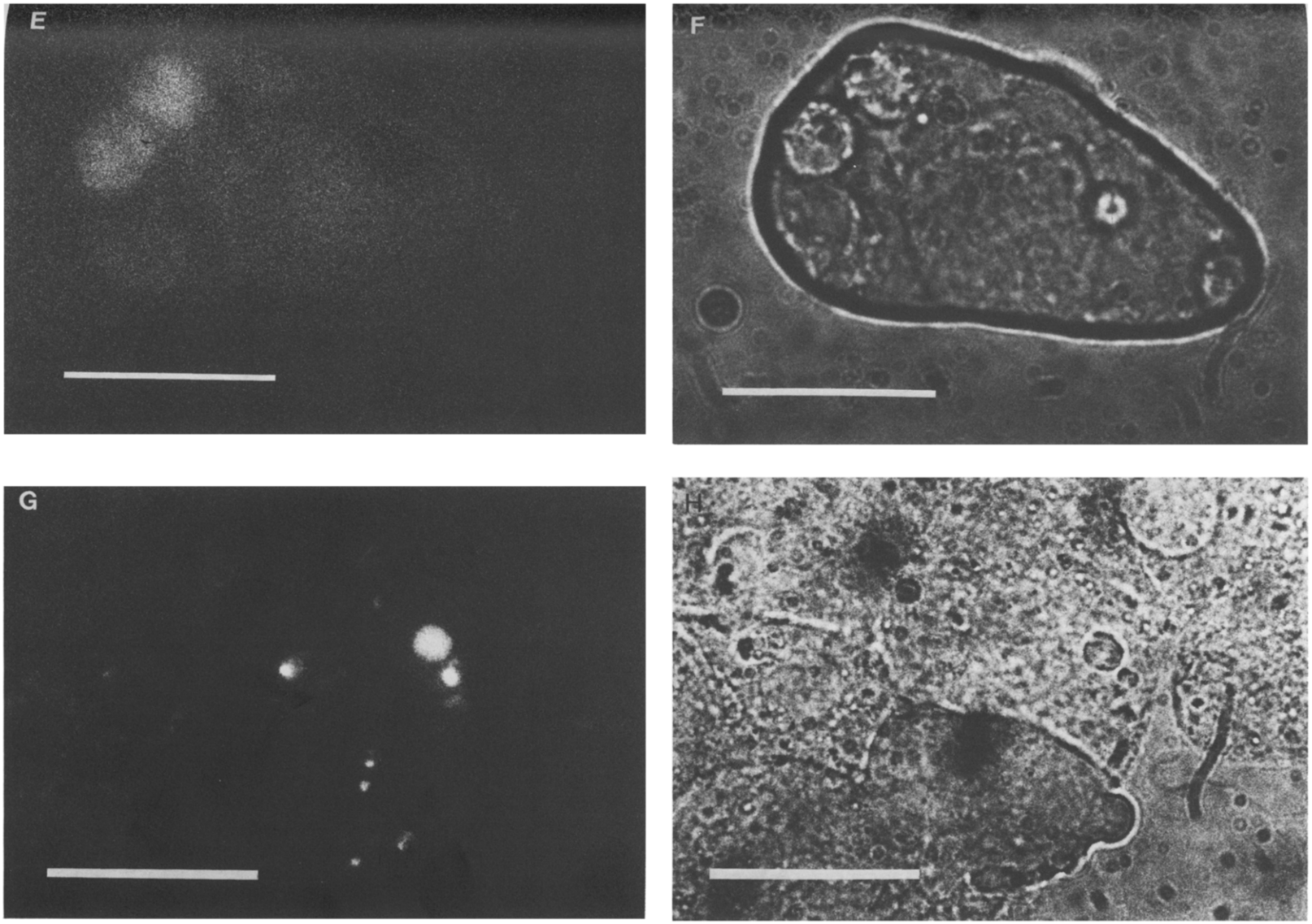


Fig. 1. (A–H) Fluorescent megamitochondria in mouse hepatocytes. Hepatocytes were isolated from cuprizone-treated mice one hour after intravenous injection of liposome-encapsulated AO-DNA. Fluorescence images, obtained using a Zeiss Universal microscope equipped with a standard filter for green excitation, are shown beside their analogous phase contrast images. Fluorescence photographs, taken prior to phase contrast, required exposure times of about three minutes, and led to frequent cell mortality. Arrows point out areas of dye concentration within mitochondria (A, C). In (E) two fluorescent megamitochondria are visible. Contours of the adjacent nonfluorescent nucleus in the cell center are visible (F). Bars represent 10 μm.

TABLE I

SIZE DISTRIBUTION OF MEGAMITOCHONDRIA

Normal rat liver mitochondria have an average diameter of 1.1 μm. Nuclei have diameters of 8.1 ± 1 μm. Peroxisome average diameter is 0.6 μm [20].

Method of visualization	Diameter (μm)
1. Light microscope, whole cell images	0.9 to 3.5
2. Light microscope, isolated mitochondria	1.9 to 6.7
3. FACS, fluorescent populations	2.6 (3 min) 3.7 (30 min, major) 5.9 (30 min, minor) 3.0 (60 min) 3.0 (60 min)
4. Transmission EM, tissue thin sections	0.5 to 4.2

3. Flow cytofluorimetry of megamitochondria

Results from the FACS analysis of megamitochondria are shown in Figs. 2A and 2B and Tables II–IV.

Aliquots from suspensions of isolated megamitochondria used in flow cytofluorimetry were examined by transmission EM after negative staining with ammonium molybdate (pH = 7.4). The mitochondria were mostly intact and displayed the spherical shape characterizing mitochondria as opposed to the ruffled appearance of mitoplasts [7,25].

Histogram 2A shows results from the liver of a mouse 30 min after intravenous injection of 0.150 mg of liposome-encapsulated AO-DNA. A population of relatively highly fluorescing particles of a size appropriate to be megamitochondria is seen, for analysis divided into two populations, having diameters of 3.7 μm and 5.9 μm. 23000 particles of a size greater than 1 μm were counted. For size comparison, a star indicates the position of 3 ± 0.1 μm beads at this instrument setting. A similar relatively highly fluorescing population appeared in FACS analyses of megamitochondria isolated from mouse liver excised 3 min after injection as well as in two experiments at 60 min after injec-

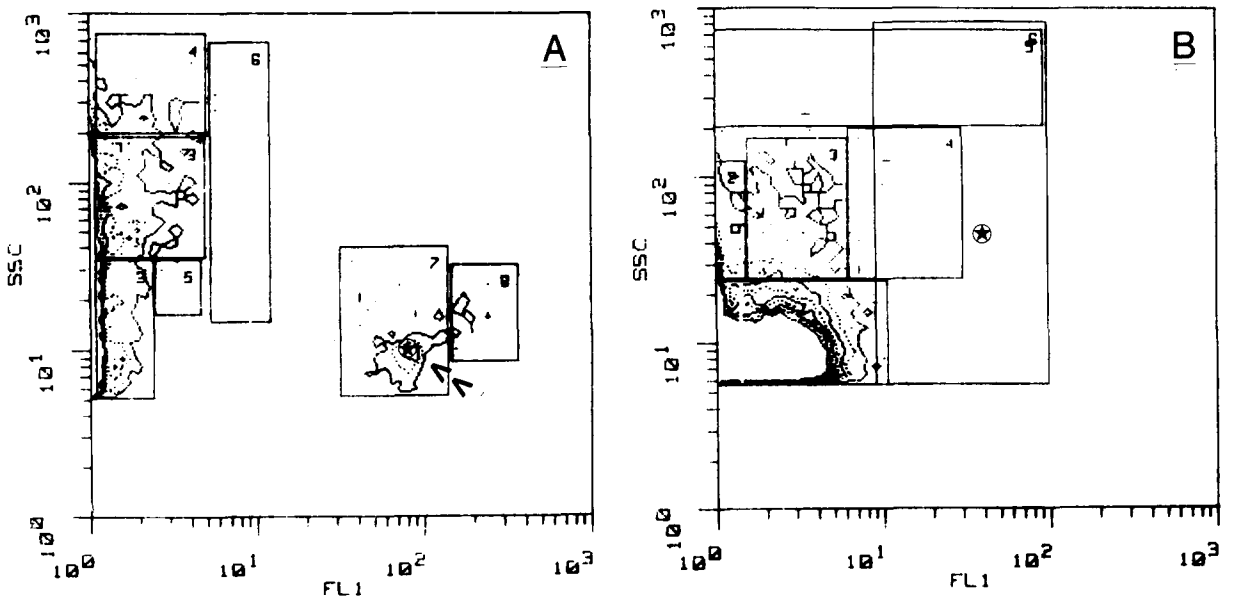


Fig. 2. FACS analysis of isolated megamitochondria. Histogram (A) resulted from analysis of megamitochondria isolated 30 min after intravenous injection of 0.150 mg of liposome-encapsulated AO-DNA complex. About 23000 particles of a size greater than about 1 μm were counted. Characteristics of the fluorescent populations No. 7 and No. 8 are listed in Tables III and IV. For size and fluorescence intensity measurements, polystyrene sulfonate beads of 3 ± 0.1 μm and 6 ± 0.1 μm diameter and known fluorescence intensity were analyzed at the same time using the same instrument setting. The position of 3 ± 0.1 μm beads is denoted by a star. Histogram (B) resulted from analysis of megamitochondria isolated one hour after intravenous injection of 0.150 mg of free AO-DNA complex. 50000 particles of a size greater than about 1 μm were counted. The position of 3 ± 0.1 μm beads at this instrument setting is denoted by a star.

TABLE II

FACS: COMPARISON OF ALL POPULATIONS OF A SINGLE ANALYSIS

Megamitochondria were isolated from mouse liver excised 3 min after intravenous injection of liposome-encapsulated AO-DNA.

Population No.	Average particle size (μm)	% of total particles counted	Average relative fluorescence intensity [a]
1	1.9	96.94	$8.7 \cdot 10^{-3}$
2	2.6	0.07	4.06
3	8.5	1.55	$7.4 \cdot 10^{-3}$
4	10.7	1.15	$1.7 \cdot 10^{-2}$
5	25.6	0.14	$7.0 \cdot 10^{-2}$
6	60.7	0.03	$2.2 \cdot 10^{-2}$

[a] Values have been normalized to the fluorescence intensity of the injected liposome suspension (Materials and Methods).

tion of liposome-encapsulated AO-DNA (Tables III and IV).

Histogram 2B shows the sizes and fluorescence distributions of particles in a suspension of megamitochondria isolated from the liver of a mouse one hour after intravenous injection of 0.150 mg of free AO-DNA. No population having significant fluorescence is observed. 50000 particles of size greater than about 1 μm were counted. For size comparison, the position of 3 ± 0.1 μm beads

TABLE III

FACS: COMPARISON OF THE MOST HIGHLY FLUORESCENT POPULATION IN EACH ANALYSIS

I. Experiment, time after injection	II. Size, average diameter (μm)	III. % of counted particles	IV. Relative fluorescence of megamitochondria [a]
Liposome-encapsulated AO-DNA			
3 min	2.6	0.07	4.06
30 min	3.7	11.46	64.13
	5.9	2.22	161.91
60 min	3.0	1.35	58.95
60 min	3.0	2.40	65.54
Free AO-DNA			
60 min	2.7	1.03	0.01
	3.0	1.17	0.08
	5.5	0.02	0.23

[a] Values have been normalized to the fluorescence intensity of the injected liposome suspension (Materials and Methods).

at this instrument setting, slightly different from that of Histogram 2A, is designated by a star.

The 'uptake' of dye by megamitochondria, calculated as the percentage of AO-DNA injected per liver cell that was later contained by a mitochondrion, increased throughout the 60 min after injection which were investigated, as listed in Column V of Table IV. Dye concentration was

TABLE IV

FACS: COMPARISON OF QUANTITIES OF AO-DNA CONTAINED BY MEGAMITOCHONDRIA

I. Experiment, time after injection	II. mmoles AO [a] injected per liver cell ($\times 10^{14}$) [b]	III. Relative fluorescence of megamitochondria [c]	IV. mmoles AO [a] in a 3 μm mitochondrion ($\times 10^{18}$) [d]	V. % (AO-DNA in a mito/ AO-DNA injected per liver cell) [a]
Liposome-encapsulated AO-DNA				
3 min	2.3	4.06	3.72	0.02
30 min	37.8	64.13	58.77	0.02
		161.91	148.39	0.04
60 min	1.7	58.95	54.03	0.32
60 min	1.7	65.54	60.07	0.35
Free AO-DNA				
60 min	31.8	0.01	0.01	0.0
		0.08	0.07	0.0
		0.28	0.25	0.0

[a] Ratio DNA/AO = $4 \cdot 10^{-4}$:'1, see Paragraph 2, Materials and Methods.

[b] Mice were weighed prior to injection. We assumed $650 \cdot 10^6$ cells in the liver per 100 g body weight [30]. An uptake of 60% of the injected dose by the liver was assumed [26].

[c] Values have been normalized to the fluorescence intensity of the injected liposome suspension (Materials and Methods).

[d] The mitochondrion was assumed to be a sphere. Mitochondrial volume = $1.42 \cdot 10^{-11}$ ml.

measured, and a DNA : Acridine orange ratio of $4 \cdot 10^{-4} : 1$ was assumed (Materials and Methods). Calculations of dye injected per liver cell were made assuming a 60% uptake of total injected dose by the liver [26]. At 3 minutes after injection, an average of 0.02% of the AO-DNA injected per liver cell was contained by a fluorescent mitochondrion. At 30 minutes, the fluorescent population could be divided into two subpopulations for analysis: one population (11.46% of counted particles, Table III) contained 0.02% of AO-DNA injected per liver cell and a second population (2.22% of counted particles) contained 0.04% of AO-DNA injected per liver cell (i.e., 14 and 28 molecules of DNA, respectively). This value increased to 0.32% in one experiment at 60 min and 0.35% in another experiment at 60 min. Thus at 60 min after injection, a megamitochondrion which was fluorescent contained an average of 0.33–0.34% of the AO-DNA which was injected per liver cell. The increase of percentage probe injected which is associated with a megamitochondrion corresponds with our earlier observation by EM autoradiography that the concentration of liposome-encapsulated DNA associated with mitochondria increases over 1 hour [2].

The molar concentration of dye in mitochondrial populations was determined indirectly from the relative fluorescence of megamitochondria by spectrofluorimetric comparison against AO-DNA solutions of known concentration (Materials and Methods). A volume was then calculated for a hypothetical average megamitochondrion, assuming a spherical shape and a diameter of 3 μm. Thus the mmoles of dye contained by such a megamitochondrion could be calculated and are listed in Column IV of Table IV.

The relative fluorescence values of the megamitochondria are listed in Column III of Table IV. The values have been normalized to the fluorescence intensity of the injected liposome suspension (Materials and Methods).

In the comparison of fluorescence associated with isolated mitochondria, a normalization was made to correct for the different animal weights (i.e. liver sizes and presumably different numbers of megamitochondria). This normalization consisted of an estimation of the number of liver cells in each animal and expression of the dosage as mmoles of dye injected per liver cell, as listed in Column II of Table IV.

The fluorescent mitochondria at 60 minutes were clearly single populations, showing little scatter in fluorescence or size. The histogram at 30 min appeared more complex (Fig. 2A) and was analyzed as two populations. The average properties of the populations differed as to relative fluorescence of the megamitochondria, size, and percentage of counted particles, as listed in Tables III and IV. A high percentage of counted particles, 5-times higher than other fluorescent populations measured, having the smaller diameter of 3.7 μm, similar to the fluorescent populations at 3 min and 60 min, contained about the same percentage of AO-DNA injected per liver cell as the population at 3 min. The other population of mitochondria at 30 min, about 1.5-times larger in diameter, contained a higher percentage of dye, intermediate between 3 min and 60 min. These represented a lower quantity of counted particles, having the same order of magnitude as those observed at 60 min.

Average diameters of fluorescent megamitochondria and the percentages of counted particles they represent are listed in Table III. A fluorescent population having a diameter of about 3 μm appeared in every case. As percentages of counted particles (particles having diameters greater than about 1 μm), fluorescent megamitochondria varied from 0.07% to 2.40%, with one population of 11.46%. Thus between 3 min and 60 min, the number of fluorescent mitochondria as percentage of counted particles increased 35-fold, from 0.07% to 2.40%. The fluorescent mitochondria were about the same size in both experiments, 2.6 μm and 3.0 μm. As percentages of counted particles, populations having about the same size but containing negligible fluorescence, were 97% (3 min), 41% (30 min), and 65% and 66% (60 min, two experiments) (data not shown).

Large contaminating populations of small vesicles were excluded from counting or analysis. On the other hand, small populations of size greater than or approximate to the megamitochondria were analysed. Their sizes were appropriate for contaminating normal mitochondria (1.1 μm), nuclei (8.1 μm), and whole cells (21 μm) [20]. These populations displayed a negligible flu-

orescence in all cases. Table II shows results from the suspension of megamitochondria isolated 3 min after intravenous injection of liposome-encapsulated AO-DNA, in terms of average particle size, average relative fluorescence intensity, and the percentage of all particles counted which that population represents. The largest population, 97% of all particles counted and having a size appropriate for slightly enlarged mitochondria, does not have an unusual relative fluorescence intensity. Only a small percentage, 0.07%, having a size appropriate to be megamitochondria, shows a high fluorescence intensity (4.06).

The negligible fluorescence associated with mitochondria one hour after injection of free AO-DNA indicates the importance of liposomes in mediating the localization of encapsulated materials to mitochondria. 0.150 mg of free AO-DNA was injected, three to six times more AO-DNA than was injected in the liposome-encapsulated form, with the exception of the experiment at 30 min. Yet fluorescent mitochondria appeared in the FACS analysis only after intravenous injection of liposome-encapsulated complex. We do not exclude the possibility that free AO-DNA complex and/or free dye may have found its way to the mitochondria rapidly and was then rapidly released. But it is clear that the fluorophore associated with megamitochondria one hour after intravenous injection of liposome-encapsulated AO-DNA does not derive from contaminating free AO-DNA.

4. Electron microscopy showing the interaction of megamitochondria with liposomes encapsulating iron dextran

Transmission electron microscopy was used to support results from fluorescence experiments. Liposomes encapsulating iron dextran were found to associate with megamitochondria in mouse hepatocytes by 10 min after intravenous injection (Figs. 3A–C). This is in agreement with our previous observations for intravenously injected liposomes encapsulating ferritin, uranyl acetate, and DNA which was nick-translated with ^{125}I-dCTP, analysed using EM autoradiography and subcellular fractionation [1,2].

We wanted to observe ultrastructural details of the interaction of liposomes with megamitochondria to find whether the appearance was the same as in the case of liposome association with normal mitochondria in mouse liver. The ultrastructure of our megamitochondria resembled those previously documented [3–6], showing that the cristae configuration is generally the same as for normal mitochondria but the matrix space is larger. Normal rat liver mitochondria have an average diameter of 1.1 μm [20]. The mitochondria presented here have lengths greater than 2.3 μm. Mitochondria having larger and smaller dimensions were observed in this tissue.

As a liposome marker, we encapsulated an electron dense synthetic iron dextran having the trade name 'Gleptosil' [27]. This iron dextran is reported to contain 200 mg of iron per ml, as the iron oxide, coated with dextran. The resulting uncharged rod-shaped structure appears to be about 250 Å in length. When encapsulated in liposomes, it is trapped in the aqueous lumen of the liposome and also seems to be entrapped between the lipid multilayers of the liposome. The inset in Fig. 3A shows liposomes prior to injection. Liposomes were uni- and oligolamellar and ranged in size from 1250 Å to 2500 Å, as determined by measurement of transmission electron micrographs [28].

The stability of liposomes encapsulating such an iron dextran is not documented, and minor amounts of non-encapsulated iron dextran which were consistently present after filtration in increasing amounts over prolonged time periods are thought to be the result of leakage from liposomes during storage. However, we believe that this does not invalidate our transmission EM results, because the difference in intrahepatic fate of free iron dextran and liposome-encapsulated iron dextran was clear. Apparently similar to colloidal carbon and ferritin in catabolic fate in the liver [29], free iron dextran was concentrated in vacuoles, presumably lysosomes and phagolysosomes, in sinusoidal cells one hour after intravenous injection (Fig.4). No significant accumulation in hepatocytes was observed at this time after injection. On the other hand, liposome-encapsulated iron dextran was found in parenchymal cells in association with mitochondria, by 10 min after intravenous injection.

The electron microscope images shown in Figs.

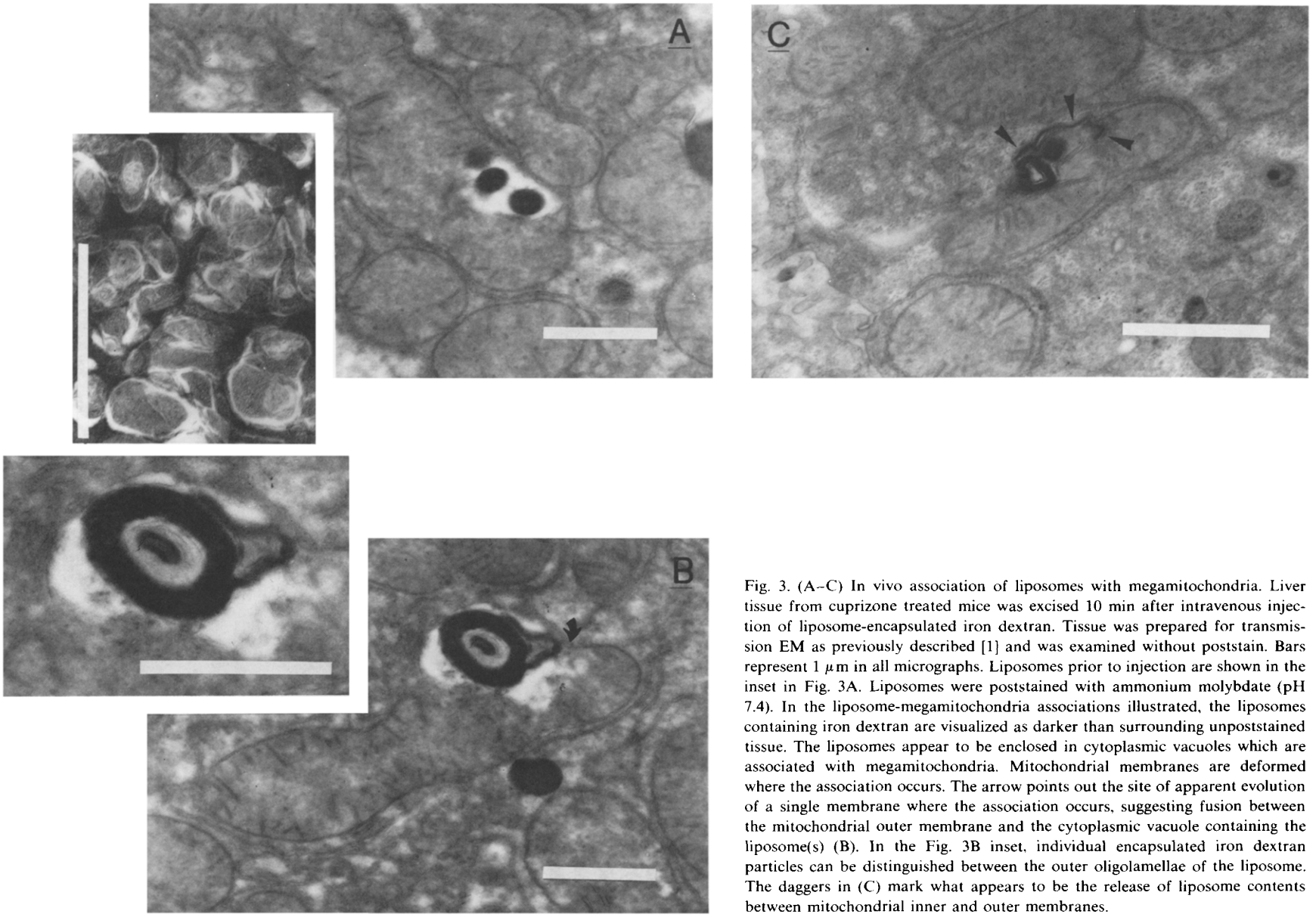


Fig. 3. (A–C) In vivo association of liposomes with megamitochondria. Liver tissue from cuprizone treated mice was excised 10 min after intravenous injection of liposome-encapsulated iron dextran. Tissue was prepared for transmission EM as previously described [1] and was examined without poststain. Bars represent 1 μm in all micrographs. Liposomes prior to injection are shown in the inset in Fig. 3A. Liposomes were poststained with ammonium molybdate (pH 7.4). In the liposome-megamitochondria associations illustrated, the liposomes containing iron dextran are visualized as darker than surrounding unpoststained tissue. The liposomes appear to be enclosed in cytoplasmic vacuoles which are associated with megamitochondria. Mitochondrial membranes are deformed where the association occurs. The arrow points out the site of apparent evolution of a single membrane where the association occurs, suggesting fusion between the mitochondrial outer membrane and the cytoplasmic vacuole containing the liposome(s) (B). In the Fig. 3B inset, individual encapsulated iron dextran particles can be distinguished between the outer oligolamellae of the liposome. The daggers in (C) mark what appears to be the release of liposome contents between mitochondrial inner and outer membranes.

3A–C display the same features as in the case of liposomes encapsulating uranyl acetate or ferritin in association with mitochondria in normal mouse liver [1]. The liposomes in the cell are visualized as darker than surrounding tissue (they contain iron dextran), and they appear to be encapsulated in vacuoles. In some cases, a single continuous membrane appeared to be formed between the mitochondrial outer membrane and the membrane of the cytoplasmic vacuole containing the liposome, suggesting fusion (arrow). The daggers in Fig. 3C mark what may be interpreted as release of liposome contents between mitochondrial inner and outer membranes. It is not possible to determine by electron microscopy whether the liposome contents are delivered to the mitochondrial inner matrix.

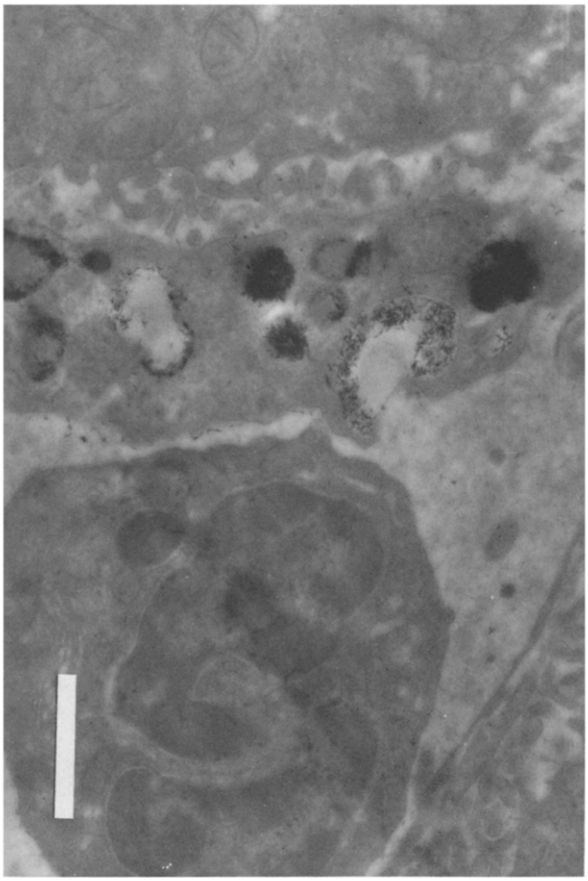


Fig. 4. In vivo localization of free iron dextran in the liver. Liver tissue from cuprizone treated mice was excised one hour after intravenous injection of 2 μg of free iron dextran (100 μl of a 1:10 dilution of 'Gleptosil', 20% iron). Tissue was prepared for transmission EM as previously described [1] and was examined without poststain. Bar represents 1 μm. The iron dextran had localized in vacuoles in sinusoidal cells, shown here. No significant iron dextran was found in parenchymal cells at this time.

The study reported here supports and extends our earlier work by showing that the apparent liposome-mitochondria interaction observed by EM is not merely a juxtaposition of liposome and mitochondrion. The fluorescence photographs and FACS analyses show unequivocally that after intravenous injection, liposome contents are later contained by isolated hepatocyte mitochondria, an assertion which could not be made on the basis of our earlier work. We support results from fluorescence experiments by transmission EM documenting the similarity between the association of megamitochondria and normal mitochondria with liposomes in vivo.

One unresolved question from this work is whether the apparent association of endocytotic vesicles (containing liposomes) with mitochondria is of fundamental importance to the mechanism of intracellular trafficking following endocytosis. We estimate from the FACS analysis that an average fluorescent mitochondrion in the liver may contain as much as 0.34% of the encapsulated material which was injected per liver cell, that is, 14 to 28 DNA molecules, in our experiments. This quantity indicates a traffic which cannot be dismissed as trivial, a priori. Further, the increase in the amount of liposome-encapsulated material associated with mitochondria over one hour means the possibility that mitochondria may serve as repositories cannot be excluded. On the other hand, we do not know whether the route of endocytotic vesicles (in our experiments, containing liposomes) to mitochondria is general regardless of vesicle content or whether this transport may be induced by some parameter such as size. We do not know whether the association is a random, statistical event or results from a vectorial traffic. However, in so far as we or others attempt to deliver macromolecules to the mitochondria using liposomes, it is irrelevant whether vesicular traffic to mitochondria is vectorial or random.

Another major question which remains is whether a mitochondrial gene transported by liposomes into hepatocytes may be expressed by

mitochondria. Experiments aimed at answering this question are under way in our laboratory.

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