Liposome Concentration in Canine Ischemic Myocardium and Depolarized Myocardial Cells

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SUMMARY To determine whether liposomes (microscopic phospholipid vesicles) may be useful in delivering drugs to a region of myocardial ischemia, we studied the concentration of positively charged and neutral liposomes containing \(^{131}\)I-albumin and horseradish peroxidase in ischemic myocardium of 20 dogs during the first 4 hours of experimental myocardial infarction. We also studied the interaction of liposomes containing fluorescent dyes and horseradish peroxidase with isolated contracting cardiac myocytes. We found that positively charged and neutral liposomes accumulated in poorly perfused myocardium and that positively charged liposomes accumulated in the ischemic region to a greater extent than neutral liposomes (138 ± 21 vs. 81 ± 9\% (mean ± SE) of the concentration of liposomes in uninvolved myocardium). Electron microscopic examination of this myocardium showed liposome contents to be located in the vascular space, in endothelial cells, and in ischemic myocytes. We found that isolated cardiac myocytes avidly took up the liposomal contents when they were depolarized by a high potassium environment and that liposomal contents were scattered throughout the interior of the cells in electron micrographs of some of the isolated myocytes. Anoxia alone for 20-30 minutes did not modify the liposome-isolated myocyte interaction or cause depolarization of the cells. We conclude that liposomes may be useful as drug carriers to depolarized, ischemic myocardium, although significant uptake by normal myocardial cells cannot be expected with the lecithin, cholesterol, and octadecylamine liposomes we used.

A MAJOR impediment to the drug therapy of myocardial infarction is the limited access that drugs have to ischemic myocardium. All present methods of delivering drugs to an ischemic area are dependent on myocardial blood flow to that area, which always is impaired. To investigate a mode of delivery with the potential to transport drugs to an ischemic region in greater proportion than blood supply might ordinarily allow, we studied the specialized carrier, liposomes. We investigated the interaction of liposomes with isolated contracting cardiac myocytes and the fate of liposomes in the hearts of dogs with experimental myocardial infarction.

Liposomes are microscopic vesicles made up of a bilayer phospholipid membrane surrounding an aqueous phase. Drugs can be incorporated in liposomes within the aqueous phase or, possibly, within the lipid phase, as well. The uses and properties of liposomes have been reviewed extensively; they have been employed by several investigators as drug carriers (Gregoriadis, 1976; Tyrrell et al., 1976). There is little known, however, concerning the interaction of liposomes with ischemic or normal myocardial tissue. Caride and Zaret (1977) reported that positively and neutrally charged liposomes, but not negatively charged liposomes, concentrate in the region of an experimental myocardial infarction 24 hours after coronary embolization. Their findings suggest that liposomes could be used to transport drugs to the region of the myocardial infarction in concentrations that could not be achieved by standard methods of drug administration.

Although the results of their experiment are very provocative, there are some methodological difficulties with this work, and there are many important additional questions which require investigation. The marker that Caride and Zaret used for their liposomes is a small molecule, \(^{99m}\)Tc-DTPA (diethylene triamine pentaacetic acid). Small molecules are known to leak from liposomes (Jonah et al., 1975; Tyrrell et al., 1976). Furthermore, \(^{99m}\)Tc-DTPA is known to concentrate in a myocardial infarction when unassociated with liposomes, and was considered for use in nuclear medicine as an infarct scanning agent (Davis et al., 1976). The marker that we used has a lesser tendency to concentrate in a region of myocardial is-
In our experiments we investigated several additional important questions. We examined the interaction of liposomes with isolated, contracting cardiac myocytes. We wished to know whether these cells, which would be the major target of potential therapeutic interventions using liposomes, could take up the contents of liposomes, and which factors present in ischemic myocardium would influence the cell-liposome interaction. In dogs with a coronary ligation, we investigated the uptake of positively charged and neutral liposomes by normal and ischemic myocardium using labeled albumin as an aqueous phase marker. In addition, we determined the uptake of free albumin by ischemic myocardium compared to normal myocardium. By studying the first 4 hours of myocardial ischemia, we looked at the most critical period of the course of myocardial infarction. In this same model, we used electron microscopy to localize liposomal contents in normal and ischemic myocardium.

**Methods**

### Liposomes

**Preparation of Liposomes**

Neutral and positively charged liposomes were prepared with the use of the lipid concentrations shown in Table 1A. Solutions of the lipids in 10 ml of chloroform were evaporated to dryness at 30°C under a stream of dry nitrogen. The lipid material was resuspended in 5 ml of hexane and dried again under nitrogen. Liposomes were made that contained aqueous phase solutes as shown in Table 1B. Six to 10 ml of the aqueous solution were added to the lipid material and the mixture was sonicated by use of a Labsonic 5-mm ultrasonic probe at a 20-40 watt setting. The temperature of the mixture was held near 35°C during sonication with a water bath that was, at the onset of sonication, about 25°C. Optical clarity was achieved in 30–45 minutes, and sonication was continued for an additional 1 hour in order to produce a population of small, unilamellar liposomes. The relatively long period of sonication necessary to achieve optical clarity may be related to the water bath temperature having been at or below the lipid mixture transition point. Temperatures near the ultrasonic probe likely were above 35°C after sonication was in progress for 45 minutes. Finally, the liposomes were annealed at 40°C for 30 minutes (Lawaczek et al., 1976).

### Separation of Liposomes from Non-Encapsulated Medium

All liposome preparations were filtered through a Millipore filter (0.45 μm), loaded onto a 3 x 33 cm glass column containing Sephadex G150 equilibrated with 0.9% NaCl, and eluted with saline. The eluate was collected in 2-ml aliquots. To identify visually the liposome fraction, we added 0.3–0.5 ml of 0.5 M fluoresceinate dye to the aqueous solution before sonication. Additionally, 0.1 ml aliquots of the elution fractions were diluted with 2.5 ml of distilled water and the absorbance at 492 nm (absorption peak of the dye) was measured to locate precisely the liposome fraction. Between 0.5 and 2.0% of the labeled albumin was entrapped within the liposomes.

### Capacity of Liposomes to Retain Labeled Albumin

To determine the leakage of labeled albumin from liposomes (permeability of liposomes to labeled albumin), we incubated neutral and positively charged liposomes containing 125I-human serum albumin in fresh dog plasma for 2, 5, or 24 hours at 37°C. After incubation, the liposome and plasma mixture was filtered through a Millipore filter (0.45 μm) and placed on a 1.5 x 33 cm Sephadex G150 column. The column was eluted with saline and the eluate was collected in 0.5-ml aliquots. The radioactivity in each fraction was counted in a Beckman 300 well-type γ counter. The amount of radioactivity not found within the fraction identified as containing liposomes (between 2.6 and 3.8%) was considered to be the proportion of labeled albumin that had leaked from the liposomes (see Table 2). Less than 0.1% of the radioactivity of such a liposome suspension stored in 0.9% saline for 7 days at room temperature was outside the liposome fraction.

### Freeze-Fracture Electron Microscopy of Liposomes

A small drop of 10% liposome suspension was placed on a specimen carrier, frozen in liquid Freon-22, and stored in liquid nitrogen. The material was fractured at -110°C at 2 x 10⁻⁶ mbar and immediately shadowed with platinum-carbon at 45°C fol-
TABLE 2  Percent Leakage of \( ^{31} \)I/Albumin from Liposomes

<table>
<thead>
<tr>
<th>Exposure time at 37°C*</th>
<th>2 hours</th>
<th>3 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral liposomes</td>
<td>2.60%</td>
<td>2.92%</td>
<td>3.18%</td>
</tr>
<tr>
<td>Positively charged liposomes</td>
<td>2.98%</td>
<td>3.83%</td>
<td></td>
</tr>
</tbody>
</table>

* Incubated in fresh dog plasma.

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The replicas were cleaned with sodium hypochlorite, washed in four changes of distilled water, and collected on uncoated grids. The replicas were examined and photographed with a Hitachi Hu-12 electron microscope. In the six batches examined, liposomes were 1–9 lamellar vesicles with diameters ranging from 0.04 to 3.0 μm (Fig. 1), although the vast majority were of the smaller size (0.04–0.07 μm in diameter).

**FIGURE 1** A: Freeze-fracture electron micrograph of neutral liposomes. The majority of liposomes are about 0.05 μm in diameter. B: Freeze-fracture electron micrograph of liposomes showing that one large liposome is composed of at least nine membranes.
Isolated Myocytes

myocardial cells were dispersed with trypsin, where (Marvin et al., 1979). Briefly, ventricular study had spontaneous contraction frequencies of approximately 80 beats/min.

The interaction of liposomes with individual cardiac myocytes was studied, using isolated cells from 2- to 4-day-old rats. The methods used for isolation and culture of the cells is described in detail elsewhere (Marvin et al., 1979). Briefly, ventricular myocardial cells were dispersed with trypsin, counted, diluted, and grown for 3 days on polylysine-coated 9 × 22 mm glass coverslips in petri dishes containing HEPES-buffered M199. At the time of use, the cells were single cells, small groups, or interconnected networks and strands. Confluent sheets of cells were not used. Cells selected for study had spontaneous contraction frequencies of 2- to 4-day-old rats. The methods used for isolation and culture of the cells is described in detail elsewhere (Marvin et al., 1979). A coverslip with attached cells was placed in a 300-μl chamber (Jim's Instrument Manufacturing, Inc.) to allow constant suffusion with isotonic solution (ISM) at 37°C. When we wished to expose the cells to liposomes, the liposomes were added to the beaker of ionic solution suffusing the cells or added as a 30-μl pulse from an Eppendorf pipette. The ratio of liposome suspension to suffusion medium ranged from 1:50 to 1:1000 in the known dilutions.

Fluorescence Observations and Microphotometry

Cells were observed through incident light illumination from a mercury arc lamp with a Ploemopak system, using 390- to 490-nm excitation with a 515-nm suppression filter. Photomicrographs were made with an Orthomat W photon counting camera. Photometry was carried out by using a Leitz MPV Compact photomultiplier with shutters and a peak reader, using a 30-msec flash of light for measurement to avoid fluorescence fading phenomena. All photometry was carried out during a measured steady state of fluorescence intensity.

Studies with Fluorescent Dyes

Liposomes contained sodium fluoresceinate (Fisher) or 6-carboxy fluoresceinate (Eastman) dyes. Neither of these dyes rapidly crosses the myocardial cell membrane. The latter dye has the special property of being strongly self-quenching when entrapped within the liposomes, making it useful to show delivery of liposome contents to the cell interior (Weinstein et al., 1977). Ten batches of liposomes containing fluoresceinate (6 neutral, 4 positive), and 16 containing 6-carboxy fluoresceinate (10 neutral, 6 positive), were used in these experiments. Thirty-eight different batches of isolated myocytes were used. In each experiment, myocytes were suffused with medium containing liposomes or free dye (no liposomes) for 5–20 seconds, and then with medium alone for 5 minutes. They then were examined for fluorescence. As an additional control, the cells were exposed to suffusion medium containing the fluoresceinate or 6-carboxy fluoresceinate, unassociated with liposomes for 180 seconds.

Liposome Entry during Anoxia or in Depolarizing Solution

To determine whether anoxia increased the uptake of liposomes by isolated myocytes, we suffused contracting myocytes in a gas-tight, temperature-controlled, Silastic and glass chamber (Jim's Instrument Manufacturing, Inc.) on the stage of the microscope. The cells first were placed in an oxygen-rich or anoxic environment for 20 minutes by suffusion with medium gassed with 95% O2 and 5% CO2 or with 100% N2. During this period, the cells suffused with the oxygen-rich medium continued to beat at a regular rate of about 120 beats/min. The anoxic cells, on the other hand, decreased their rate to between 30 and 55 beats/min, and their contractions became markedly irregular. A separate set of cultures was exposed to 100 mM K+ solution with Na+ decreased by 100 mM to maintain isotonicity (ISK 100) for 10 minutes. The K+ depolarized cells stopped all contraction. After the equilibration period, the cells were suffused with oxygen-rich, anoxic, or depolarizing medium containing liposomes. After 5–30 seconds of liposome exposure, the suffusion medium was changed only by eliminating liposomes, and the cells examined for any visible fluorescence during the next 15 minutes.

Horseradish Peroxidase Localization of Liposome Contents

Rat cardiac cell cultures were grown on Aclar coverslips (2 mm × 2 mm) in 60-mm Falcon culture dishes. A small drop of cell suspension, at a density of 2 × 10⁴ cells/ml, was placed over each coverslip, with nine coverslips in each culture dish. After 3–5 days, coverslips were removed from the culture dishes and rinsed twice (at 37°C). They then were exposed to a 10% liposome suspension for 2 seconds, rinsed briefly (at 37°C), and immediately immersed in a fixative containing 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.36, at room temperature. After 1 hour, the coverslips were washed in 0.1 M cacodylate buffer and stored overnight at 7°C. The coverslips were incubated for 10 minutes at room temperature in a 5 mg/ml solution of 3,3′-diaminobenzidine in 0.05 M Tris HCl buffer, pH 7.6, containing 0.01% H2O2. They then were washed in three changes of distilled water, postfixed in 2% OsO4, for 1 hour, dehydrated in an ethanol series, and embedded in Spurr's low viscosity epoxy resin. Thin sections were cut with a Sorvall MT2-B ultramicrotome and examined with a Hitachi Hu-12 electron
microscope. Even though the reaction product was clearly visible without further staining, the sections were stained with uranyl acetate and lead citrate to increase the contrast of the cellula structure. Two control samples were included in which the cells were treated and prepared identically to the procedures described above, except for one step. In one control, solution lacking liposomes was substituted for the 10% liposome suspension. In the other control, H$_2$O$_2$ was omitted from the 3,3'-diaminobenzidine solution.

**Intact Animal Studies**

**Preparation**

These studies were carried out in 29 mongrel dogs of either sex weighing between 16 and 27 kg. The dogs were anesthetized with chloralose, 50 mg/kg, and urethane, 500 mg/kg, iv. and ventilation was maintained with a Harvard animal respirator. A cannula was placed in a femoral artery for pressure measurement and for withdrawal of arterial blood samples for microsphere measurements and blood gas determinations. A femoral venous catheter was inserted for injection of further doses of anesthetic, as necessary, and for injection of liposomes or free albumin. A left thoracotomy was performed, and the heart was exposed. A catheter was placed in the left atrium for pressure measurement and for microsphere injections. The left anterior descending coronary artery was ligated just distal to its first diagonal branch, and visible collaterals to this region were ligated also. One hour after ligation, liposomes containing radioactively labeled albumin or labeled albumin unassociated with liposomes were injected iv. Eleven dogs received neutral liposomes, nine received positively charged liposomes, and nine received albumin unassociated with liposomes. Immediately afterward, total and regional myocardial blood flow was measured with radioactively labeled microspheres. Five minutes after the injection of liposomes or free albumin and every 30 minutes during the next 3 hours, blood samples were taken to determine liposome or free albumin concentration. Three hours after injection of the liposomes or albumin, the dogs were killed with an injection of saturated potassium chloride solution.

The left anterior descending coronary artery was cannulized at the site of ligation, and Evan's blue dye was injected at a pressure equivalent to the mean arterial pressure recorded during the experiment. This served to mark the ischemic region. The heart then was removed. The right ventricle, atria, large epicardial coronary vessels, epicardial fat, fibrous skeleton of the heart, and valvular structures were trimmed away from the left ventricle. The ventricle then was sliced every 3 mm perpendicular to its long axis. The sections within 1 cm of the apex were discarded because the borders of the blue-stained region were never clear, and in the remaining sections, the blue-stained regions were cut out carefully with a sharp scalpel blade. A 4-mm wide transmural segment was taken from the center of the blue region and another from the edge of the blue region. These pieces were, in turn, cut into epicardial, midwall and endocardial thirds. Sections also were taken from the posterior-lateral wall of the left ventricle, which was not stained with Evan's blue dye.

**Measurement of Blood Flow with Microspheres and Determination of Labeled Albumin Concentration**

Myocardial perfusion was measured with carbonized microspheres, 15 μm in diameter, labeled with $^{40}$Sc, $^{85}$Sr, or $^{14}$Ce. Between 1 x 10$^5$ and 2 x 10$^5$ microspheres, suspended in less than 2 ml of saline, were injected into the left atrial cannula during a period of 10 seconds, and the cannula immediately was flushed with 5 ml of saline. Starting 30 seconds before the injection of microspheres and continuing for 90 seconds after injection, blood was withdrawn through the arterial catheter at a rate of 2.06 ml/min with a Harvard pump. Prior to injection, the vial containing the microspheres and 1 drop of Tween-80 was vigorously agitated mechanically for at least 4 minutes. Microscopic examination of spheres agitated in this manner showed that in excess of 98% were dispersed completely.

After completion of the study, the heart was cut as described above. The pieces of the same category, e.g., central infarct-subepicardium, were combined, weighed to the nearest milligram, and placed in plastic tubes. The reference blood samples and the blood samples taken for measurement of albumin or liposome concentration also were placed in plastic tubes. Each tube was counted for 4 minutes in a well-type sodium iodide γ scintillation counter. Standard techniques were used for isotope separation (Heymann et al., 1977).

Myocardial blood flow was determined using the formula: \[ BF_m = \frac{(C_m \times 100 \times BF_r)}{Cr} \] in which \( BF_m \) = myocardial blood flow (ml/min x 100 g); \( C_m \) = counts per gram in the myocardium; \( BF_r \) = reference blood flow (rate of withdrawal from the reference artery); and \( C_r \) = total counts in the reference blood.

**Location of Liposome Contents in the Myocardium**

To determine the location of liposomal contents in the myocardium, we injected liposomes containing horseradish peroxidase iv in dogs 1 hour after coronary ligation. The same protocol was used as described above. Neutral or positively charged liposomes were injected into two dogs. At the end of the experiment, the myocardium was inspected. In all four of the dogs, a cyanotic portion of myocardium that bulged in systole could be identified. The heart was removed quickly and subendocardial and
subepicardial samples of about 0.5 g were taken from the center and edge of the ischemic region and from normal myocardium. The tissue was cut into small pieces and fixed in glutaraldehyde buffered with sodium cacodylate. It was washed with Tris-hydrochloric acid buffer containing dianinobenzenidine and then exposed to OsO₄ buffered with sodium cacodylate. The tissue was dehydrated with ethanol and embedded in Spurr's plastic. Thin sections were cut and examined with the Hitachi electron microscope.

Statistical Analysis
Data are expressed as the mean ± standard error of the mean. Student's t-test for paired or unpaired data and analysis of variance were used where appropriate. Differences were considered significant when P was less than 0.05.

Results
Studies with Isolated Cardiac Myocytes
Depolarization Increase of Liposome Uptake

After exposing cardiac myocytes to neutral or positively charged liposomes containing fluoresceinate for 5–20 seconds, only faint fluorescence of these cells was visible. Non-muscle cells, which have low (about −15 mV) membrane potentials, filled with more fluorescence than muscle cells. No fluorescence was observed in cells exposed to fluoresceinate in the absence of liposomes. After membrane depolarization with 100 mM K⁺, fluorescence strong enough to photograph was found in muscle cells (Fig. 2). The cells depolarized (to −12 ± 0.08 mV) by 100 mM K⁺ took up about five times more dye as normally polarized cells (−76 ± 0.6 mV) (Fig. 3), and the depolarized cells took up the dye more rapidly. The positively charged liposomes were more rapidly taken up than neutral liposomes. Depolarizing solution consistently enhanced uptake of both neutral and positively charged liposomes, as reflected by greater fluorescence.

Lack of Effect of Anoxia on Liposome Uptake
Myocytes exposed to 20 minutes of anoxia had the same small amount of fluorescence as cells in an oxygen-rich environment. The experiment was conducted five times each with neutral and positively charged liposomes containing 6-carboxy fluoresceinate, and the results were no different with

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**Figure 2.** Liposomes concentrated in isolated cardiac muscle cells only when the cell membrane had depolarized. A: Differential interference contrast photomicrograph (×800) of a contracting cell from which action potentials (B) were recorded. Only fluorescence too weak to photograph could be seen. C: Fluorescence micrograph with KP500/TK510 filter showed the same cell filled with fluorescence after depolarization of the cell by 100 mM K⁺. D: The cell then had a membrane potential of −5 mV and showed no anodal break responses.
Exposure Time, sec.

FIGURE 3 Uptake of liposomes is shown by fluorescence measured by photometry at 5-, 10-, 15-, or 20-second exposure times. Dye accumulated rapidly in the cells in all cases during the first few seconds and then accumulated more slowly as time progressed. Dye concentration was low enough to prevent quenching. The depolarizing ISK100 solution (isotonic solution with potassium concentration of 100 mM) consistently resulted in greater dye uptake. O = neutral liposomes and △ = positive liposomes. Fluorescence values are arbitrary units. ISM = isotonic solution for mammals with normal potassium concentration.

or without anoxia. Resting membrane potential was −75 ± 1.8 mV in the anoxic cells, not different from the cells in oxygenated solution. The only obvious effect of the anoxia was a change from a regular contraction pattern of about 80/min to an irregular pattern of about 35/min.

Horseradish Peroxidase

With this experiment we investigated the subcellular location of liposome contents. Following exposure of isolated contracting myocytes to liposomes containing horseradish peroxidase, we found horseradish peroxidase reaction product present as small electron-dense particles spread throughout the myocytes (Fig. 4). No similar electron-dense material was present in the controls processed without liposomes or without H₂O₂ in the 3,3’-diaminobenzidine solution. We could see no quantitative or qualitative differences between cells exposed to neutral or positively charged liposomes, and no differences could be detected among cells incubated for 2 seconds, 30 seconds, 10 minutes, or 30 minutes. All the exposed myocytes contained the reaction product. Therefore, liposomes rapidly interacted with the myocytes, depositing their contents throughout the cells.

Intact Dog Experiments

Concentration of Liposomes in Ischemic Myocardium

Table 3 contains the perfusion data and liposome or free labeled albumin concentrations for the three groups of dogs. The central ischemic zone was perfused at less than 11% of the blood flow to the normal myocardium, and the peripheral zone at less than 35%. Perfusion of the uninvolved myocardium was comparable in the three groups. Liposome or free albumin concentration in the ischemic zone is presented as a percentage of concentration in normal myocardium. Positively charged liposomes concentrated to the greatest degree, and their concentration was significantly different from that of free albumin. The ratio of subendocardial to subepicardial blood flow was greater than one in the normal myocardium, but significantly lower in both the central and peripheral ischemic zones. The endocardial-epicardial ratios of the two types of liposomes or of free albumin were comparable in the normal myocardium and near unity. In the center of the ischemic zone, the endocardial-epicardial ratio for the positively charged liposomes was greater than that for free albumin.

There was little difference in the clearance of neutral and positively charged liposomes and of free albumin from the blood over a 3-hour period. Since we have shown the liposomes do not leak albumin and that they distribute differently in the heart than free albumin, this similarity in blood clearance is probably fortuitous (Fig. 5).

Localization of Liposomal Contents in Myocardial Tissue

Electron micrographs of sections of ischemic and normal myocardium taken 3 hours after injection of horseradish peroxidase-containing liposomes showed the presence of reaction product in and near the vascular spaces (Fig. 6). The ischemic myocardium was identified easily by the presence of swollen and disrupted mitochondria, loss of glycogen granules, and the presence of cellular edema. The reaction product within myocardial cells was found within ischemic myocytes. On high power micrographs, the bilayer membrane could be identified surrounding the reaction product. In some micrographs (Fig. 6C) small, irregularly shaped densities appeared which were similar to those seen in isolated cells, and we believe that this material was horseradish peroxidase reaction product from disrupted liposomes. In general, more reaction product was seen in sections from ischemic myocardium than from normal myocardium.

Discussion

Our experiments have shown that positively charged liposomes concentrated in a region of myo-
cardiac ischemia within the first 4 hours following coronary occlusion. Electron microscopic studies showed that the liposome contents were located mainly in the intravascular and interstitial spaces, in endothelial cells or in ischemic myocytes. We also have shown that isolated contracting myocytes can take up liposomal contents into their cytoplasm. We examined the effects of anoxia and cellular depolarization on the uptake of liposomes by isolated myocytes. Anoxia for 20 minutes does not enhance or retard this uptake. However, depolarization of the cell membrane with 100 mM K⁺ enhances uptake of both neutral and positive liposomes by at least 5-fold. In this section, we will discuss several implications of these findings and then speculate on future directions for the use of liposomes in the cardiovascular system.

That liposomes can deliver their contents into the cytoplasm of cardiac myocytes was demonstrated by the uptake of fluorescent dyes. Two mechanisms have been suggested to explain uptake of liposomal contents. One is endocytosis, an active energy-dependent process (Huang and Pagano, 1975), and the other is fusion, which is energy-independent (Batzri and Korn, 1975). Although the data here cannot be used to select unequivocally between mechanisms, fusion with a dependence on surface charge would be consistent with our data.
Neutral liposomes, free albumin, and positively charged liposomes were shown during the course of the experiments in dogs with coronary artery ligation.

Neutral liposomes, free albumin, and positively charged liposomes were shown during the course of the experiments in dogs with coronary artery ligation.

It seems possible, but less likely, that depolarized cells would actively transport the liposomes. Greater uptake by cells with smaller electric fields was also shown by the non-muscle cells in the cultures. These cells could be identified easily as non-muscle by lack of electrical excitability, and are always present in primary cultures (Marvin et al., 1979). The cell culture experiments and intact dog experiments both showed this liposome uptake into endothelial and non-muscle cells, which would represent an important part of the distribution of liposomes injected into intact animals.

Our results in the intact dog studies agree qualitatively with those of Caride and Zaret (1977), but we found different degrees of liposome concentration in the ischemic zone. Positive liposome concentration was about 130% of that in normal myocardium, whereas they found a concentration of between 300 and 500% of normal. By using an aqueous phase marker which is a large protein and does not, by itself, concentrate in an ischemic region to a greater extent than the liposomes, and by annealing the liposomes (Lawaczeck et al., 1976) to further prevent leakage, we developed an appropriate experimental tool to test whether liposomes can concentrate in ischemic myocardium. Furthermore, we studied the early, most critical period of ischemia from the standpoint of therapeutic intervention. Our experimental design included testing the leakiness of our albumin-containing liposomes and using our aqueous phase marker, unassociated with liposomes, as a control. The disparity between our results and those of Caride and Zaret can probably be explained by a leakage of some DTPA, an infarct-avid substance, from their liposomes during the course of the experiment. Also, the difference in time after coronary occlusion when liposome uptake was studied—the first 4 hours vs. the 24th through 28th hours—may have influenced the results.

The mechanism by which liposomes accumulate in an ischemic region is unknown. We believe that their ability to accumulate in poorly perfused regions of myocardium may be partially non-specific, and related to their size and surface charge, and not only to their liposome structure. There are several lines of evidence that suggest this may be so. We found that free albumin, unassociated with liposomes, was present in the ischemic region to a much greater extent than would be expected if its presence were proportional to blood flow. The ratio of free albumin concentration in the normal myocardium to the ischemic region was about 1:1, whereas the ratio for blood flow was about 10:1. Kloner et al. (1977) found that colloidal carbon particles, approximately the same size as liposomes, accumulate in the region of an experimental myocardial infarc-
These particles were found primarily in and around damaged blood vessels. Furthermore, drugs accumulate against a flow gradient in ischemic myocardium. Young et al. (1976) found that [\(^{3}H\)dexamethasone concentrates in a region of myocardial ischemia at 79% of the concentration found in normal myocardium, even though blood flow in this region was presumably markedly reduced. In their experiment, the drug was given iv at the onset of ischemia and assayed 2 hours later. In a report by Wenger et al. (1977), procainamide concentration was measured in normal and ischemic regions of the left ventricle, as defined by microsphere measurements of blood flow. In ischemic myocardium (<10% of normal zone flow), the procainamide concentration was 31-40% of that in normal myocardium. They infused procainamide iv during a period of 4 hours following coronary occlusion. Thus, substances of large and small molecular weight (colloidal carbon, albumin, dexamethasone, procainamide) accumulate to varying degrees in regions of myocardial ischemia, but the degree of accumulation is less than that which we observed for positively charged liposomes.

There are a number of exciting potential uses for liposomes as drug carriers to ischemic, depolarized myocardium. We should begin by studying whether drugs carried by liposomes have a desired localized pharmacological effect compared to drugs administered in a conventional manner, using sensitive parameters such as electrophysiological indicators, regional wall motion, or regional coronary vascular resistance. More precise targeting of liposomes might be possible by complexing antimyosin antibody fragments to the membrane (Gregoriadis and Neerunjun, 1975). It might be possible to test whether substances thought to act through receptors on the cell surface have an effect in the cellular interior. It also would be possible to deliver substances, such as cyclic AMP, which do not readily
cross the cell membrane, to individual contracting myocytes. Thus, liposomes will have increasing potential for use in cardiovascular research as more details about their interaction with the various cells in the heart are understood.

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