Investigations of a New, Highly Negative Liposome with Improved Biodistribution for Imaging

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An attractive feature of liposomes is the wide range of lipid composition that can lead to liposome formation, coupled with the observation that liposome biodistribution may be altered by varying lipid composition. For instance, adding charged lipids to neutral lecithin will alter the biodistribution of the resulting charged liposomes. We have prepared highly negative liposomes by replacing lecithin with negatively charged cardiolipin. The liposomes have been labeled in the lipid phase with Ga-67 and Tc-99m oxine and their properties evaluated. The expected high negative charge of the resulting liposomes was confirmed by an ion-exchange chromatographic technique. Using paper chromatography, the stability of the label was determined during incubation in saline and serum. Finally, biodistributions were determined at 2 hr in mice, and the results compared with those for negative lecithin liposomes. Accumulated activities in liver and spleen were reduced by factors of five and 20, respectively, over lecithin liposomes. Since preferential accumulation of activity in these organs constitutes the biggest limitation to the use of lecithin liposomes, cardiolipin liposomes may prove to be more useful carriers of radioactivity in imaging applications. More importantly, however, these results illustrate the value of studying novel liposome types as potential radiopharmaceuticals.


Although liposomes have been investigated in detail for applications such as drug transport and cell-membrane studies, their potential in nuclear medicine has not been fully established. Several studies have investigated the biodistributions of gamma-emitting radionuclides contained in either the aqueous or the lipid liposome phase (1–15).

All of these previous studies have used liposomes composed mainly of phosphatidylcholine (lecithin) with a small amount of cholesterol to improve stability. Since lecithin is an uncharged zwitter-ion at neutral pH, small amounts of charged lipids, such as dicetyl phosphate, have been added to provide a surface charge. Studies with these charged liposomes have demonstrated a small but significant effect of charge on biodistribution (10, 12–14).

A major attraction of liposomes is the almost unlimited variation possible in the composition of the liposome membrane. Since surface charge has been shown to be a determinant of biodistribution, it is reasonable that increasing liposome charge through the use of novel lipid compositions may increase the effect on biodistribution. This may be best accomplished not by adding small amounts of charged lipids to lecithin, but rather by replacing lecithin with a charged lipid capable of forming liposomes.

We have prepared liposomes composed primarily of cardiolipin, a molecule similar in structure to lecithin but possessing a double negative charge at neutral pH, as
shown in Fig. 1. The liposomes have been labeled in the lipid phase with both Ga-67 oxine and Tc-99m oxine. The relative surface charges of these liposomes have been measured by an anion-exchange column technique. In addition, the stability of the labeled liposomes has been determined in both saline and serum environments. Finally, biodistributions of the Ga-67 liposomes has been determined in normal mice at 2 hr and the results compared with those for negative lecithin liposomes.

**MATERIALS AND METHODS**

**Liposome preparation.** Liposomes were prepared by standard methods. Neutral liposomes were composed of lecithin (L-α-phosphatidyl choline dipalmitoyl) and cholesterol (molar ratio 7:1); conventional negative liposomes were composed of lecithin, dicetyl phosphate, and cholesterol (molar ratio 7:2:1); and positive liposomes were composed of lecithin, stearylamine, and cholesterol (molar ratio 7:3:2). Cardiolipin liposomes were composed of cardiolipin (diphosphatidyl glycerol) and cholesterol (molar ratio 7:2). Stearylamine,* cardiolipin,† and the remaining chemicals‡ were obtained from commercial sources and were used without further purification.

Gallium-67 was obtained as the citrate radiopharmaceutical. Benzyl alcohol present in the radiopharmaceutical extracts into chloroform, along with the oxine complex, and is not removed during rotary evaporation in the preparation of liposomes. Therefore, it was first removed by extraction into chloroform. The pH of the Ga-67 citrate solution was then adjusted to about 5 by the addition of 2 ml of 0.05 M sodium acetate buffer, pH 5.6, to the 4-ml aqueous phase. The oxine complex was then formed by the addition of 100 μl of an ethanol solution of oxine containing 2 mg/ml of oxine.‡ The Ga-67 oxine complex was then extracted into two 2-ml volumes of chloroform. The extraction was usually repeated with 50 μl of oxine solution and the chloroform extracts combined. Normally about 50-80% of the Ga-67 activity was recovered.

Technetium-99m was obtained as pertechnetate from a generator. The oxine complex was prepared by adding together 1 ml of a fresh, nitrogen-purged solution containing 50 μg of SnCl2·2H2O and 100 μl of the above oxine solution. The pH was then adjusted to between 8–9 with dilute NaOH, and 1–3 mCi of pertechnetate solution added. The oxine complex was extracted with 2–3 ml of chloroform. The recovery ranged between 70 and 90%.

Approximately 10 mg of the appropriate lipids were dissolved in 4–6 ml of chloroform in a round-bottom flask and mixed with either the Ga-67- or the Tc-99m-oxine chloroform solution. The flask was then connected to a rotary evaporator and the contents evaporated in 30–45 min under reduced nitrogen pressure while the flask was maintained at 60°C in a water bath. Following evaporation, 1 ml of either 0.9% NaCl (pH 7.5) or 0.05 M PBS (pH 7.3) was added to the flask and the lipids coating the walls dispersed with a magnetic stirring bar. The flask was then placed in a bath sonicator§ for 1–10 min in the case of cardiolipin liposomes and 60 min for all other liposomes. During sonication, the liposomes were kept at or below room temperature by periodic addition of ice to the bath. Labeled liposomes were separated from free activity by chromatography.

![Structural formulae of three compounds pertaining to this research.](image-url)
on a 1.5-× 20-cm column of Sephadex G-50 using 0.05 M PBS as eluant. The milky suspension eluting near the void volume contained the radiolabeled liposome fractions. The labeling efficiency was determined by counting the liposome-containing fractions either in a NaI(TI) well counter or in an ionization dose calibrator and comparing with a standard of the unfraccionated suspension. Each preparation was inspected using a light microscope to ensure that the liposomes were uniform in size and free of aggregates.

Surface-charge measurements. Ion-exchange chromatography was investigated for the purpose of measuring relative liposome surface charge. Neutral, negative, and positive lecithin liposomes labeled with Ga-67 were applied to different column lengths of AG 1 × 4 anion-exchange resin and AG-50 × 8 cation-exchange resin. The effects of eluant composition, resin mesh size, column flow rate, etc., on the elution of activity from these columns were determined for each of these liposome types. On the basis of these measurements, an anion-exchange column was constructed to determine the relative charge of negatively charged liposomes.

Stability studies. Since cardiolipin-cholesterol liposomes have not been studied previously, a method was required for estimating the relative stability of the label during incubation in saline and serum. Paper chromatography, using Whatman No. 1 paper and saline eluant, pH 7, was used to determine the fraction of activity bound to liposomes. Negative lecithin liposomes and cardiolipin liposomes labeled with Ga-67 or Tc-99m were incubated in 1:1 dilution of saline or fresh human serum with frequent agitation for up to 2 hr at both 20 and 37°. Samples were taken for paper chromatography at 5 min and 1 and 2 hr. In all cases, after spotting, the papers were not permitted to dry before chromatography. This stability assay not only determined the fraction of activity still bound to liposomes, but also determined the chemical form of the released activity.

Biodistributions. In this study mice were killed 2 hr after injection, to ensure that liposome degradation, with release of label to the circulation, does not contribute significantly to the distribution. Cardiolipin liposome preparations were used for biodistribution determinations immediately after swelling and gel chromatography. Lecithin liposomes were occasionally stored overnight in a refrigerator before use, since stability studies demonstrated that only slight dissociation occurred under these conditions.

Approximately 1 mg of lipids in 0.1–0.2 ml of saline containing about 5 μCi of activity was administered by tail vein to each CD-1 male mouse. Following etherization of each animal at 2 hr, a blood sample was obtained by cardiac puncture and tissue samples were removed for weighing and subsequent counting in a NaI(TI) well counter. Samples of bone were obtained from the intact femur. Results were obtained for Ga-67 negative lecithin and cardiolipin liposomes, and for Ga-67 oxine itself; results are expressed as percentage injected dose per gram wet weight, normalized to a 25-g mouse. In the case of Ga-67 oxine, the activity was administered in 50% ethanol.

To ensure that the distributions observed were not influenced significantly by unidentified variables of the preparations, two preparations of each liposome type were studied and the results are reported separately.

RESULTS

The average Ga-67 labeling efficiencies were 15, 80, and 60% for neutral, negative, and positive liposomes, respectively, and 10% for cardiolipin liposomes. The Tc-99m labeling efficiency for cardiolipin liposomes was similar. Although all lecithin liposome types appeared to be uniform in size on the basis of light microscopy, the particle-size distribution of the cardiolipin liposomes appeared to be smaller. This was confirmed by centrifugation measurements, in which 20% of the activity in a cardiolipin liposome preparation would typically be centrifuged under specific conditions, compared with 45% for lecithin liposome preparations. In order to prepare cardiolipin liposomes with size distributions similar to those of the other liposome types, sonication time was reduced to between 1 and 10 min. The same fraction numbers from G-50 chromatography were collected for use.

An electron-microscopic examination of a cardiolipin liposome preparation was achieved by mixing 1 drop of an unfraccionated liposome preparation (swelled in

FIG. 2. Electron micrograph of a cardiolipin liposome.
distilled water rather than saline) with 1 drop of a 2% phosphotungstate solution and allowing the mixture to stand for 20 min. It was then evaporated to dryness on a copper grid and placed in an electron microscope. Figure 2 is an electron micrograph obtained in this manner; it clearly shows the multilaminar structure of the liposome.

As mentioned previously, the relative liposome surface charge was determined by ion-exchange chromatography. Neutral, negative, and positive lecithin liposomes labeled with Ga-67 were applied to columns of anion- and cation-exchange resins. A 50–100-mesh size was found to be suitable and was chosen to reduce the likelihood of physical trapping. Figures 3 and 4 illustrate the behavior of the liposomes on 1.5- × 20-cm columns at a flow rate of 20 sec/ml. In Fig. 3, results of passing neutral, negative, and positive liposomes through the anion-exchange AG 1 column are shown; negative liposomes are eluted to a negligible degree relative to neutral and positive liposomes. The reverse is true on the cation exchanger AG 50, as shown in Fig. 4. Surprisingly, it is not the position of the peak that is affected by liposome charge, but rather its magnitude. A possible explanation assumes that liposomes of appropriate charge have strong affinity for the resin's binding sites, and these sites are easily saturated. Activity would then elute from the column only if the charge and numbers of binding sites are appropriate. The fact that neutral liposomes and liposomes with the same charge as the resin are not eluted completely is possibly due to the zwitter-ion nature of lecithin. In support of these suggestions, we have observed that the degree of elution for all liposome types may be increased by shortening the columns or by increasing the flow rate.

Additional evidence that these phenomena are due to effects of liposome charge was obtained by measuring the degree of elution of negative liposomes from an AG 1 column with eluants at different pH. The results of this study are shown in Fig. 5. The degree of elution is constant at neutral and basic pH but increases with decreasing pH below about 4. This behavior is easily explained, since dicetyl phosphate—the compound that provides the negative charge on these liposomes—has a pK_a between 1 and 2 (16). Consequently as the pH of the eluant approaches 2, the liposome will begin to lose its negative charge and be eluted as neutral. This study was performed both with distilled water and with 0.9% NaCl eluent. As expected, the presence of NaCl did not affect the shape of the curve, but did increase the degree of elution at each pH value. This effect is due to the competition between chloride ions and liposomes for the resin's binding sites.

To quantitate the surface charge of negative liposomes, a 1.5- × 5-cm AG 1 column was prepared and used with distilled-water eluants, pH 7, and at the same flow rate. The column was calibrated with radiolabeled neutral liposomes (lecithin and cholesterol, molar ratio 7:2), negative liposomes (lecithin, dicetyl phosphate, and cholesterol, molar ratio 7:2:1) and “half-negative” liposomes (lecithin, dicetyl phosphate, and cholesterol, molar ratio 7:1:1). The percentage of activity eluted near the void volume was determined for each of these preparations. The results appear in Fig. 6; each datum is the average of at least three determinations, usually on more than one preparation. The bars show the ranges of measurements. The charge density of the liposomes, based on the molar percentage of the dicetyl phosphate (and assuming a charge of 1.5 per molecule at neutral pH), is plotted on the abscissa relative to that of negative lecithin liposomes. The curve shows the expected dependency of percent elution on liposome charge. Cardiolipin liposomes with a charge density of 2.16 displayed a 10 ± 5% recovery under these conditions, confirming the expected high negative charge of these liposomes.

**FIG. 3.** Percentages of neutral, negative, and positive lecithin liposomes per fraction eluting from anion-exchange column.
Paper chromatography, on Whatman No. 1 paper and saline pH 7 eluants, was found to be suitable for measuring the loss of label from Ga-67 and Tc-99m liposomes. Being insoluble, activity bound to liposomes does not migrate from the origin in this system. Activity present in water-soluble forms travels from the origin, and may therefore be distinguished from activity present on liposomes. However, both Ga-67 and Tc-99m may exist in aqueous solution as insoluble and colloidal oxides or hydroxides, and their presence will contribute to the origin activity peaks. Since each liposome preparation was purified by gel chromatography, however, these impurities will be absent; both Ga(OH)₃ and colloidal Tc-99m (probably TcO₂) were found either to bind irreversibly to G-50 columns or to elute slowly and not along with the liposomes. Furthermore, preparations of these insoluble forms of Ga-67 and Tc-99m (as well as the oxine complexes) were incubated in serum at 37° in the usual manner and chromatographed. The percentage of activity at the origin in these chromatograms varied between 1 and 8% over the 2-hr incubation.

Figure 7 shows typical radiochromatograms obtained for Ga-67 lecithin and cardiolipin liposomes for preparations incubated in saline and serum at two temperatures. In the case of Ga-67, the chemical form of the soluble activity released from the liposomes is thought to be a colloidal Ga oxine displaying an Rf value in this system of about 0.09 (soluble Ga-67 oxine has an Rf of 0.42). In the case of Tc-99m, the principal soluble chemical form following incubation in saline is pertechnetate (Rf 0.70), whereas in serum it is the oxine complex (Rf 0.55).

The percentage of activity bound to liposomes at 5 min in saline is a measure of the radiochemical purity of the

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**FIG. 4.** Percentages of neutral, negative, and positive lecithin liposomes per fraction eluting from cation-exchange column.

**FIG. 5.** Percentages of negative lecithin liposomes eluting in void volume from an anion-exchange column, as functions of eluant acidity for both 0.9% saline and distilled water.

**FIG. 6.** Effect of liposome charge on percentage of liposomes eluting in void volume from an anion-exchange column. Liposome charge density relative to that of conventional negative liposomes is plotted on abscissa.
TABLE 1. PERCENT DISSOCIATION OF Ga-67 AND Tc-99m CARDIOPLIPIN AND LECITHIN LIPOSOMES IN SALINE AND SERUM

<table>
<thead>
<tr>
<th></th>
<th>Saline 20°C</th>
<th>Saline 37°C</th>
<th>Serum 20°C</th>
<th>Serum 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga-67 lecithin liposomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>0</td>
<td>4</td>
<td>38</td>
<td>32</td>
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<tr>
<td>1 hr</td>
<td>3</td>
<td>10</td>
<td>29</td>
<td>23</td>
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<tr>
<td>2 hr</td>
<td>8</td>
<td>12</td>
<td>29</td>
<td>25</td>
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<tr>
<td>Ga-67 cardiolipin liposomes</td>
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<tr>
<td>5 min</td>
<td>11</td>
<td>0</td>
<td>31</td>
<td>39</td>
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<td>1 hr</td>
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<td>5</td>
<td>27</td>
<td>45</td>
</tr>
<tr>
<td>2 hr</td>
<td>0</td>
<td>11</td>
<td>39</td>
<td>48</td>
</tr>
<tr>
<td>Tc-99m lecithin liposomes</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>56</td>
</tr>
<tr>
<td>1 hr</td>
<td>18</td>
<td>41</td>
<td>29</td>
<td>48</td>
</tr>
<tr>
<td>2 hr</td>
<td>20</td>
<td>11</td>
<td>39</td>
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<td>Tc-99m cardiolipin liposomes</td>
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<td>5 min</td>
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<td>0</td>
<td>0</td>
<td>53</td>
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<tr>
<td>1 hr</td>
<td>25</td>
<td>43</td>
<td>6</td>
<td>79</td>
</tr>
<tr>
<td>2 hr</td>
<td>24</td>
<td>64</td>
<td>39</td>
<td>79</td>
</tr>
</tbody>
</table>

much less stable to the release of activity than are the Ga-67 liposomes.

Biodistributions were obtained only for the Ga-67-labeled liposomes, because of their greater stability. The results obtained for two preparations each of negative lecithin and cardiolipin liposomes are presented in Table 2 along with that for Ga-67 oxine itself. Clearly the biodistribution of the label has been significantly altered by changing the carrier liposome from negative lecithin liposomes to the highly negative cardiolipin liposomes. In particular, accumulation in liver has decreased by a factor of about five, while that in the spleen has decreased by a factor of about 20. In addition, blood, lung, and kidney levels have also decreased, whereas uptake in bone and muscle has increased.

By considering the relative weights of each organ and

TABLE 2. BIODISTRIBUTIONS AT 2 HR IN NORMAL MICE FOR Ga-67 OXINE AND FOR TWO PREPARATIONS EACH OF Ga-67 CARDIOPLIPIN AND NEGATIVE LECITHIN LIPOSOMES

<table>
<thead>
<tr>
<th></th>
<th>Ga-67 cardiolipin liposomes</th>
<th>Ga-67 lecithin liposomes</th>
<th>Ga-67 oxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.4 ± 1.0</td>
<td>2.9 ± 0.8</td>
<td>8.5 ± 1.3</td>
</tr>
<tr>
<td>Brain</td>
<td>0.30 ± 0.10</td>
<td>0.20 ± 0.01</td>
<td>0.22 ± 0.12</td>
</tr>
<tr>
<td>Liver</td>
<td>6.9 ± 3.1</td>
<td>5.1 ± 0.7</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.6 ± 1.7</td>
<td>5.7 ± 1.9</td>
<td>70 ± 19</td>
</tr>
<tr>
<td>Lung</td>
<td>2.2 ± 0.6</td>
<td>3.3 ± 0.7</td>
<td>7.1 ± 2.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.0 ± 0.5</td>
<td>1.7 ± 0.4</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Heart</td>
<td>2.1 ± 0.8</td>
<td>1.9 ± 0.7</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Bone</td>
<td>9.9 ± 3.1</td>
<td>7.3 ± 2.0</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.8 ± 2.5</td>
<td>1.2 ± 0.6</td>
<td>0.80 ± 0.17</td>
</tr>
</tbody>
</table>

* Results expressed as % injected dose/g normalized to unit body weight with 1 s.d. of the mean. Each datum is the average of five animals.
tissue (17), we estimate that some 60% ± 14% of the
injected activity was accounted for in the case of the mice
studies with Ga cardiolipin liposomes in contrast to 85%
± 12% for the lecithin liposomes. This difference, if
significant, may indicate that the cardiolipin liposomes
are cleared more rapidly into gut and/or urine than are
the lecithin liposomes.

DISCUSSION

Perhaps the most significant aspect of liposomes is the
almost infinite variety of lipid composition that can lead
to liposome formation. Of special interest to applications
in nuclear medicine is the possibility that certain of these
variations will effect biodistributions in a useful manner.
One property of liposomes that may be readily altered
is surface charge. Previous work in this area has dem-
onstrated an effect of charge on biodistribution, but all
of these previous studies have apparently used liposomes
composed primarily of lecithin to which small ad-
mixtures of charged lipids have been added. Liposomes
of much higher charge may be prepared by replacing
lecithin with phospholipids that are themselves charged.
For example, in this study we have used cardiolipin to
provide highly negative liposomes; other phospholipids
that may be suitable are phosphatidylerine (18),
phosphatidylglycerol (19), and phosphatidic acid.
Similarly, highly positive liposomes may be prepared
from phosphatidylglycerol lysine (19). Cholesterol has
been reported to stabilize artificial lipid vesicles (11), and
may be an important addition to these liposomes.

In a study of this nature, the properties of the new
liposome type must be carefully evaluated. Obviously,
an effort must be made to ensure that liposome forma-
tion has actually occurred and that for comparison
purposes the size distribution is similar to that of con-
tventional liposomes. It must be possible to label the
liposomes to good yield and to demonstrate that the la-
beled liposome is stable in serum environments at 37 °C.
If the object is to prepare charged liposomes, some
method must be available to measure this charge. The
ion-exchange assay used in this work is simple and pro-
vides reproducible results. An electrophoretic method
of measuring relative liposome charge has also been used
(19). It would be particularly interesting to measure the
charge following incubation in serum environments,
since the presence in serum of ions, such as Ca2+, may
affect liposome charge in vivo by binding to the liposome
surface (20). Finally, it is particularly important to en-
sure that duplicate preparations of each liposome type
provide reproducible results.

The use of the paper chromatographic assay has
permitted the determination of the fraction of activity
bound to liposomes in suspension of these particles. Of
particular interest is the observation that, immediately
following G-50 chromatography, appreciable activity
is present in a soluble chemical form, probably as the
oxine complex. Since purification on Sephadex columns
will remove these low-molecular-weight species, their
presence following chromatography must indicate that
rapid re-equilibration has occurred. Likewise, we have
shown that exhaustive equilibrium dialysis is only par-
tially successful in removing these soluble species.
Hwang (21) has shown that In-111 oxine diffuses rapidly
through the lipid bilayers of liposomes and exchanges
readily from one liposome to another. Because of its
chemical similarity to indium, Ga-67 oxine may be ex-
pected to behave in a similar manner. Results from this
study indicate that the oxine complex of Tc-99m is even
more prone to diffuse from lipid bilayers. These results
are similar to those we have obtained in a separate study
of leukocytes labeled with Tc-99m oxine; they were
found to release their label much more readily than
leukocytes labeled with Ga-67 oxine.

An important result of this study of cardiolipin lipo-
somes is the decreased accumulation of activity in liver
and spleen relative to that observed in all previous studies
with lecithin liposomes. This observation is particularly
noteworthy, since the principal limitation of liposome
use in nuclear medicine is the preferential localization
of activity in these organs. Although accumulation in
liver and spleen can be reduced, often slightly, through
the use of small liposomes (8, 13, 22), subcutaneous
and intraperitoneal administration (6), use of carrier stable
liposomes (7, 8), incorporation of specific proteins (5),
and novel methods of liposome preparation (9), these
methods are not practical in all cases.

As previously mentioned, light microscopy and cen-
trifugation measurements showed the size distribution
of the cardiolipin liposomes to be smaller than that of the
lecithin liposomes. This was so despite a much shorter
sonication time. Some decrease in liver and spleen
accumulation with decreasing liposome size is expected,
but the magnitude of this effect is far smaller than that
observed in this work, since the reduction is by a factor
of 2–4 in spleen uptake and 2 or less in liver uptake (8,
13, 21). An increase in liver uptake with decreasing
liposome size has been reported (5).

The labeling efficiency achieved in this work of about
10% for the cardiolipin liposomes is a limitation that
must be dealt with before imaging studies can be per-
formed with this agent. Furthermore, a method of la-
beling liposomes of this and other types after their
preparation, rather than during preparation as was the
case in this work would be highly desirable. A method
of labeling preformed lecithin liposomes with Tc-99m
has been reported (12), however, we were unable to form
a stable label using this method. A promising approach,
developed in our laboratory, is the incorporation of a
lipophilic compound containing a lipophobic chelating
group.
FOOTNOTES

* Pfaltz and Bauer, Inc., Stamford, CT.
1 Grand Island Biological Co., Grand Island, NY.
2 Sigma Chemical Co., St. Louis, MO.
3 Bronsonic Model 10-002, 25 watts.
4 Charles River Breeding Laboratories, Wilmington, MA.

ACKNOWLEDGMENTS

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REFERENCES


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