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# Lung Uptake of Liposome-entrapped Glutathione After Intratracheal Administration

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**Abstract**—The intratracheal delivery of glutathione (GSH), in liposome-encapsulated form, prolongs retention of the drug in the rat lung. This study has been designed to determine the extent and time-course of pulmonary tissue uptake of administered $^{14}$C-labelled liposomes containing [$^{3}$H]GSH. Liposomes, composed of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine each with or without 30 mol% cholesterol showed a clearance >80% from the bronchoalveolar lavage, 24 h after intratracheal instillation. Lung tissue content of $^{14}$C-lipid increased with time: by 72 h, 42–56% of the administered dose was recovered from the tissue. [$^{3}$H]GSH was present in pulmonary tissue in relatively constant amounts throughout the 72 h, ranging from 14–35% of the administered dose. Vesicles composed of DMPC had a low entrapment for GSH. There were only minor differences in GSH entrapment efficiency and lung tissue uptake between vesicles of the other lipid compositions.

Most investigations of liposomes as drug-delivery systems have relied on parenteral routes of administration. To achieve targeted delivery to the lung, however, the direct administration of liposomes into the airways has the advantage of circumventing systemic dilution and removal by other tissues and organs (Shek et al 1990). Moreover, extrapulmonary adverse effects may be minimized. Phospholipids are endogenous to the lung (Baxter et al 1969) and liposomes composed of naturally-occurring phospholipids, at an appropriate dose, should not pose a toxicological risk to this organ.

Direct administration of liposomes into the bronchi and alveoli has been investigated for the treatment of respiratory distress syndrome (Ivey et al 1980) and for the delivery of antitumour drugs (Juliano & McCullough 1980), antimicrobial drugs (Wyde et al 1988), and anti-asthmatic drugs (Pettenazzo et al 1989; Taylor et al 1989) to the pulmonary system. We have recently demonstrated enhanced retention in the rat lung of glutathione (GSH), after its intratracheal instillation in liposome-encapsulated form (Jurima-Romet et al 1990). However, it was not known whether the liposomes were taken up by the lung tissue, or whether the vesicles remained in the air spaces. In this study, we have determined the recovery of GSH and liposomes in rat bronchoalveolar lavage fluid (BAL) and lung tissue, up to 72 h after their intratracheal administration. Other studies have shown that the lipid composition of the carrier vesicle is an important determinant of the interaction of liposomes with cells (Poste & Papahadjopoulos 1978) and the rate of release of the entrapped agent (Szoka & Papahadjopoulos 1980). Therefore, this study has been conducted using different constituent phospholipids, dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC), with or without the inclusion of 30 mol% cholesterol.

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## Materials and Methods

*Materials*

Cholesterol, DMPC, DPPC, DSPC, and GSH were purchased from Sigma (St. Louis, MO). [$^{3}$H]GSH, Protosol tissue solubilizer, and liquid scintillation cocktails were obtained from New England Nuclear (Boston, MA). [$^{14}$C]Cholesterol was from Amersham (Arlington Heights, IL). Other reagents and solvents were of analytical grade.

*Preparation of liposomes*

Lipids were dissolved in chloroform–methanol (2:1 v/v) to which was added 20 nmol of [$^{14}$C]cholesterol containing 0·11 MBq of radioactivity. The lipid was dried, under a stream of nitrogen, to a thin film coating the inside of a glass vessel. Any traces of solvent were removed by placing the vessel under vacuum for at least 1 h. To the dry lipid, 30 nmol of [$^{3}$H]GSH containing 1·1 MBq of radioactivity was added. The lipid was then hydrated with 1 mL (per 100 mg of lipid) of 0·4 M GSH in 5 mM phosphate buffer (pH 6·5) containing 30 mM EDTA. The formation of liposomes requires the hydration temperature to exceed the gel-to-liquid-crystalline phase transition temperature of the lipid. Thus, for the entrapment of GSH in liposomes composed of DSPC, DPPC and DMPC, the GSH solution was warmed to 65, 51 and 33°C, respectively, i.e. 10°C above the transition temperature of each lipid. Multilamellar vesicles were formed by vortexing the lipid-aqueous mixture. The suspension was then frozen in liquid nitrogen and thawed in a warm water-bath. The freeze-thaw process was repeated 5 times. The preparation was transferred to an Extruder (Lipex Biomolecules, Inc., Vancouver, BC) and extruded, under helium, through 2 stacked polycarbonate filters of 400 nm pore size, 10 times. Free GSH was removed by washing the liposomes twice in 5

mM potassium phosphate buffer (pH 6·5), and pelleting at 110000 *g* for 1 h at 5°C. Supernatant and pellet samples were counted for $^{3}H$ and $^{14}C$ to determine the GSH entrapment efficiency. The liposomes were resuspended in the buffer to a final lipid concentration of 60–90 μmol $mL^{-1}$. Vesicle size distribution was determined for the final liposomal preparation with the use of a Coulter N4SD particle-size analyser.

*Administration of liposomes to animals*

Male Wistar rats, 220–280 g (Charles River Canada Inc., St. Constant, Quebec), were anaesthetized with 50 mg $kg^{-1}$ of sodium pentobarbitone intraperitoneally. The trachea was cannulated with a 9 cm length of PE-200 polyethylene tubing, with the tip of the cannula positioned approximately at the tracheal bifurcation. This cannula served to guide the introduction of a 9·2 cm length of PE-50 polyethylene tubing, attached at one end to a 250 μL glass Hamilton syringe. The liposomes were administered to the animals by intratracheal instillation using this syringe. Each rat received 100 μL of liposomal suspension, followed by 50 μL of 0·85% NaCl (saline) to rinse the syringe and tubing. Animals to be killed 1·5, 3·0, and 4·5 h after administration, had the cannula secured with sutures, the incision closed with surgical staples, and the excess cannula excised to leave a 1 cm protrusion. Rats to be killed after 24, 48 and 72 h, had the cannula removed and the tracheal incision sutured.

*Collection of BAL and tissues*

Rats were anaesthetized with sodium pentobarbitone, the trachea recannulated as necessary, and a 16-G needle, with the tip cut off, inserted into the cannula. Two 20 mL syringes were attached to the needle via a 3-way stopcock. Bronchoalveolar lavage was performed by instilling phosphate-buffered saline at 37°C into the lungs via one syringe and withdrawing lavage fluid via the other. This was repeated 10 times, using 2·5 mL of phosphate-buffered saline each time. After the lavage was completed, the lungs were excised.

*Preparation of biological samples*

The BAL was spun at 1000 *g* for 10 min to pellet any cells. The supernatant was removed and a 5 mL sample was counted for $^{3}H$ and $^{14}C$ radioactivity in 16 mL of Aquasol. The lung was minced and homogenized in 2 mL saline. Samples (0·4 mL) in duplicate, were transferred to glass counting-vials and solubilized with 1·0 mL of Protosol in a shaking water-bath at 50°C. To decolourize the samples, 0·2 mL of 30% hydrogen peroxide was added after solubilization was completed: The vials were incubated with hydrogen peroxide at 50°C for 30 min. After cooling, 15 mL of Biofluor and 0·5 mL of 1 M HCl were added. The vials were kept at room temperature (20°C) for 2–3 days to allow chemiluminescence to decay, and then counted for $^{3}H$ and $^{14}C$ radioactivity.

## Results

*GSH entrapment efficiency*

Liposomes composed of DMPC had relatively low entrapment of GSH (Table 1). The incorporation of 30 mol% cholesterol improved the entrapment efficiency to levels comparable to vesicles prepared from DPPC. Cholesterol at 30 mol%, however, did not significantly affect the GSH entrapment of vesicles composed of DPPC or DSPC, which already had entrapment efficiencies of about 15–20%.

Table 1. Effect of liposomal lipid composition on GSH entrapment efficiency.

| Lipid composition | % GSH entrapment | Mean vesicle diameter (nm) |
|---|---|---|
| DMPC | 2·9 ± 1·0 (3) | 199 ± 7 (3) |
| DMPC/cholesterol | 12·4, 14·1 | 217, 265 |
| DPPC | 14·5 ± 1·7 (5) | 325 ± 18 (5) |
| DPPC/cholesterol | 18·5 ± 2·4 (4) | 178 ± 4 (4) |
| DSPC | 21·0, 20·6 | 365, 445 |
| DSPC/cholesterol | 17·3, 18·1 | 259, 279 |

Liposomes were prepared by the extrusion technique as described in Materials and Methods. For liposomes composed of phospholipid: cholesterol, the molar ratio is 7:3. Entrapment efficiency was determined by radioactivity counting of [$^{14}C$]cholesterol-labelled lipid and [$^{3}H$]GSH. Numbers in brackets indicate the number of individual determinations, s.e.m. is reported where n = 3–5.

*Lung lavage and tissue recoveries of [$^{3}H$]GSH and $^{14}C$-lipid*

After the intratracheal instillation of DMPC liposomes, 40–45% of the administered $^{14}C$-labelled lipid was recovered from the BAL during the first 4·5 h falling to 10% by 24 h (Fig. 1A). In contrast, lung tissue recovery of $^{14}C$-lipid, after administration of DMPC liposomes, increased with time (Fig. 1B). BAL recovery of [$^{3}H$]GSH was lower than recovery of $^{14}C$-lipid, only 13% at 1·5 h and decreasing to a few percent by 4·5 h (Fig. 1A). Tissue recovery of [$^{3}H$]GSH ranged from 23–39% during 72 h (Fig. 1B).

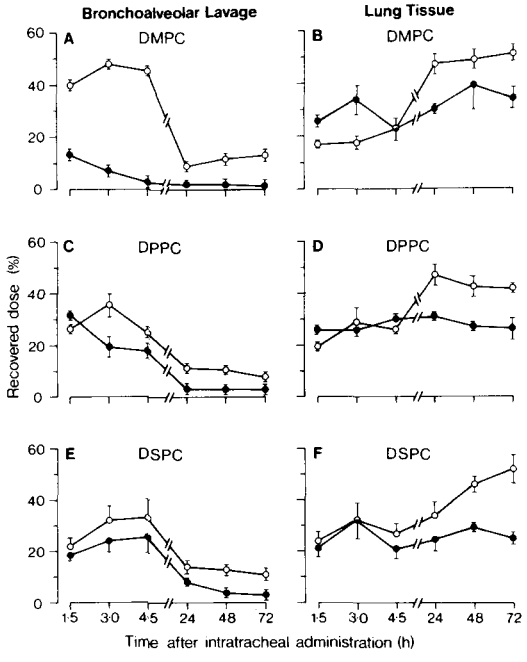


FIG. 1. Recoveries of $^{3}H$ (●) and $^{14}C$ (○) labels in rat bronchoalveolar lavage and lung tissue after the intratracheal instillation of [$^{3}H$]GSH encapsulated in $^{14}C$-labelled liposomes. Liposomes were prepared from DMPC, DPPC and DSPC, respectively, as described in Materials and Methods. Each point represents the mean percentage of recovered dose ± s.e.m. of 3–4 animals.

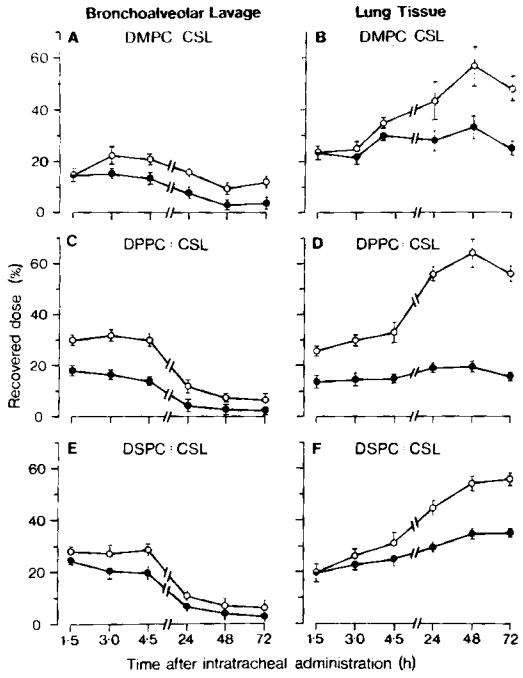


FIG. 2. Recoveries of $^{3}H$ (●) and $^{14}C$ (○) labels in rat bronchoalveolar lavage and lung tissue after the intratracheal instillation of [$^{3}H$]GSH encapsulated in $^{14}C$-labelled and cholesterol-containing liposomes. Liposomes were prepared as described in Materials and Methods from DMPC, DPPC and DSPC, each also incorporating cholesterol (CSL). The molar ratio of phospholipid:CSL in each case was 7:3. Each point represents the mean percentage of recovered dose ± s.e.m. of 3–5 animals.

Recoveries of $^{14}C$-lipid and [$^{3}H$]GSH from BAL were similar for DPPC and DSPC liposomes (Fig. 1C, E): following recoveries of $^{14}C$-lipid, ranging from 22 to 35% during the first 4·5 h, recoveries decreased to 8–11% by 72 h. The $^{3}H$ recovery curves were relatively parallel to the $^{14}C$-lipid curves at slightly lower percentage values. The observation of parallel curves for $^{14}C$ and $^{3}H$ recoveries indicate that the [$^{3}H$]GSH recovered is lipid-associated, most likely still entrapped within intact liposomes. Lung tissue recoveries of $^{14}C$-lipid, after administration of DPPC and DSPC vesicles, increased from initial levels of 20–24% at 1·5 h to 42–52% at 72 h (Fig. 1D, F). Lung tissue levels of $^{3}H$ remained relatively constant (21–31%) throughout the 72 h, indicating that the increased tissue uptake of liposomes with time did not correspond to enhancement of tissue GSH.

Generally, the observations with liposomes containing 30 mol% cholesterol (Fig. 2) were similar to the observations with cholesterol-free liposomes (Fig. 1): levels of $^{14}C$-lipid in BAL tended to decline with time, while levels in lung tissue increased with time. One apparent difference was that DMPC:cholesterol liposomes did not demonstrate the initial high recovery of $^{14}C$-lipid from BAL which was obtained with DMPC cholesterol-free liposomes (Figs 1A, 2A). The reason for the relatively higher initial recovery of $^{14}C$, from the lavage of animals instilled with DMPC liposomes, is not known.

For cholesterol-containing liposomes, the curves for [$^{3}H$]GSH recoveries from BAL were relatively parallel to the $^{14}C$-lipid recoveries (Fig. 2A, C, E); however, for lung tissue, the curves were not (Fig. 2B, D, F). For DMPC:cholesterol vesicles, [$^{3}H$]GSH in lung tissue increased only slightly with time, from an initial 23% at 1·5 h to a maximum of 33% at 48 h. By comparison, there was a much greater rise in $^{14}C$-lipid levels during this period (Fig. 2B). Similarly, for DPPC:cholesterol liposomes, [$^{3}H$]GSH in lung tissue remained constant despite a relatively large increase with time in $^{14}C$-lipid (Fig. 2D). After the administration of DSPC:cholesterol vesicles, $^{3}H$ concentrations in lung tissue increased with time, but to a lesser extent compared with the increase in $^{14}C$-lipid concentration (Fig. 2F).

Total recoveries (i.e. sum of BAL and lung tissue recoveries) of $^{14}C$-lipid and [$^{3}H$]GSH, for 1·5 and 72 h are shown in Table 2. There was a general trend of higher total $^{14}C$ recoveries at 72 h than at 1·5 h, although only a significant difference was noted for DMPC liposomes with cholesterol and for DSPC liposomes with or without cholesterol. The higher $^{14}C$ recovery may possibly be related to retention of liposomes in the upper tracheal, pharyngeal, and nasal passages shortly after their intratracheal instillation. Subsequent redistribution to lower airways occurred after the animals had recovered from anaesthesia and began to breathe and move normally. Significantly less total [$^{3}H$]GSH recovery was observed at 72 h than at 1·5 h for all liposomes except DMPC and DSPC:cholesterol vesicles. For DPPC vesicles with or without cholesterol, $^{3}H$ recoveries were markedly lower at 72 h compared with that at 1·5 h. These differences are likely accounted for by the distribution and

Table 2. Total recoveries of $^{3}H$ and $^{14}C$ from lavage and tissue of the rat lung.

| | Total $^{3}H$ recovered (% dose) | | | Total $^{14}C$ recovered (% dose) | | |
|---|---|---|---|---|---|---|
| Lipid composition | 1·5 h | 72 h | $P^{a}$ | 1·5 h | 72 h | $P^{a}$ |
| DMPC | 38·9 ± 0·8 | 35·1 ± 3·0 | ns | 57·2 ± 1·7 | 64·2 ± 2·8 | ns |
| DMPC/cholesterol | 37·5 ± 1·6 | 27·9 ± 2·8 | < 0·05 | 40·5 ± 2·1 | 63·3 ± 5·7 | < 0·01 |
| DPPC | 57·5 ± 2·4 | 28·7 ± 3·8 | < 0·01 | 46·2 ± 2·8 | 49·4 ± 2·0 | ns |
| DPPC/cholesterol | 33·1 ± 1·0 | 18·2 ± 1·1 | < 0·001 | 55·5 ± 1·5 | 62·2 ± 3·1 | ns |
| DSPC | 40·6 ± 3·8 | 27·9 ± 2·0 | < 0·05 | 45·8 ± 3·3 | 63·3 ± 5·0 | < 0·05 |
| DSPC/cholesterol | 43·3 ± 1·9 | 38·2 ± 1·4 | ns | 48·4 ± 2·0 | 61·8 ± 2·7 | < 0·01 |

The bronchopulmonary lavage fluid and lung tissue were harvested, as described in Materials and Methods, at 1·5 and 72 h after the intratracheal instillation of $^{14}C$-labelled liposomes encapsulated with [$^{3}H$]GSH. For liposomes composed of phospholipid: cholesterol, the molar ratio was 7:3. Numbers represent mean ± s.e.m. of 3–5 animals. $^{a}P$ values were computed by a non-paired Student's $t$-test comparing, for each lipid composition, the 1·5 and 72 h data points for $^{3}H$ recovery and $^{14}C$ recovery, respectively. ns, not significant.

uptake of [$^3$H]GSH by extrapulmonary tissues after release from liposomes.

## Discussion

This study has demonstrated that intratracheally instilled liposomes, composed of phospholipids with or without cholesterol, were largely cleared from the BAL by 24 h and became associated with the lung tissue. GSH, entrapped within the aqueous compartment of the vesicles, was transported to pulmonary tissues within 72 h, in amounts ranging from 16–35% of the administered dose.

GSH is unable to cross cell membranes (Puri & Meister 1983); however, it can be hydrolysed to its constituent amino acids, which are then able to translocate across cell membranes for intracellular de-novo synthesis of GSH (Hahn et al 1978). Therefore, for cells to utilize liposomally delivered GSH, either the tripeptide is released from the carrier vesicle and becomes hydrolysed extracellularly into its constituent amino acids, or the liposomes are taken up by the cells, by any of a number of mechanisms (Weinstein & Leserman 1984), and then become degraded inside the cell releasing the GSH. It is not known to what extent these mechanisms are in operation in the pulmonary environment. However, we have observed that in-vitro, rat cell-free BAL can mediate the release of GSH from liposomes in amounts ranging from 60 to 70% of the initially entrapped tripeptide for DPPC vesicles and 15–35% for DSPC vesicles over 72 h (unpublished data). Thus, extracellular release may play an important role in liposome-mediated GSH delivery. The $^3$H label, however, may represent only the constituent [$^3$H]glycine, and not the entire tripeptide molecule.

Likewise, measurement of tissue $^{14}$C does not necessarily indicate the tissue location of the intact liposome, other than that of the label; molecular exchange and transfer of lipid can occur. Exogenously administered DPPC is subject to metabolic turnover, since it is the major phospholipid component of the lung, where approximately 40% of it is associated with surfactant (Frosolono et al 1970). It has been shown that DPPC liposomes, instilled into the lungs of rabbits, associate rapidly with alveolar surfactant and enter the intracellular pool (Oyarzun et al 1980). Despite the presence of little cholesterol in alveolar surfactant, liposomal cholesterol also appears to associate with lung tissue. After the intratracheal instillation into rabbit lungs of liposomes composed of phosphatidylcholine : phosphatidylglycerol : cholesterol (5:1:4 molar ratio), Pettenazzo et al (1989) reported that approximately 20% more labelled cholesterol than labelled DPPC was found in lung tissues by 24 h, although their clearance curves from BAL were superimposable.

Whereas a bilayer marker alone does not indicate the intactness of liposomes, the determination of bilayer/internal-space-marker ratios does provide a good indication of liposomal integrity (Mayhew & Papahadjopoulos 1983). In the present study, with DPPC and DSPC vesicles, the observation of relatively parallel curves for $^3$H and $^{14}$C recoveries in BAL over 72 h, and in the lung tissue for up to 4·5 h, suggests that the liposomes were intact. After 4·5 h, despite continued uptake of the lipid-associated label, the loss of parallelism with $^3$H recovery suggests that GSH was released from destabilized vesicles.

In a previous study, we determined the recovery of radioactivity from different lobes of the rat lung after intratracheal instillation of liposomal GSH (Jurima-Romet et al 1990). The instillate was found to distribute among the 5 lobes. The distribution, however, was not uniform and could vary by as much as four-fold between different lobes. A question arises as to why total recoveries of $^{14}$C-lipid label were only 40–57% of the administered dose even as early as 1·5 h (Table 2). It is likely that immediately after intratracheal instillation, some liposomes were removed by mucociliary clearance and transported to pharyngeal and nasal passages. Liposomes may also have been engulfed by alveolar macrophages. These cells have been recovered in lung lavage in varying yields (Brain & Frank 1968). Hence, it is not known to what extent phagocytosis by alveolar macrophages may have contributed to the relatively low, total $^{14}$C recoveries. In this study, we have also measured radioactivity recoveries from cell-free BAL fluid to eliminate a possible confounding effect of different cell yields from different animals.

Individual phospholipids undergo a transition from a gel phase to a liquid-crystalline phase at a characteristic temperature. DMPC liposomes (transition temperature 23°C) were in the liquid-crystalline phase at body temperature, whereas DPPC and DSPC liposomes were in the gel state. The permeability of vesicles to entrapped compounds is greater at temperatures above the transition temperature. This point is well illustrated by our present observation that DMPC vesicles behave differently from DPPC and DSPC vesicles in BAL by a more rapid and extensive release of $^3$H label (Fig. 1A). Cholesterol reduces the permeability of phospholipid vesicles which are in the liquid-crystalline state (Szoka & Papahadjopoulos 1980). In this study, cholesterol-containing DMPC liposomes appeared less leaky, in releasing the entrapped GSH in the BAL fluid, than did cholesterol-free DMPC liposomes. This appearance is indicated by the absence of parallel elimination of the dual markers ($^{14}$C and $^3$H) for DMPC liposomes (Fig. 1A) and the presence of parallel elimination for DMPC: cholesterol liposomes (Fig. 2A). Thus, cholesterol may be useful in improving the stability of certain liposomal formulations.

The overall differences between DPPC and DSPC vesicles, with or without cholesterol, appeared to be minimal. Therefore, in the selection of lipid composition for liposomal delivery to the lung, considerations should be given to technical factors related to liposome preparation. The high transition temperature of DSPC requires the preparation of the liposomes at a relatively high temperature. Depending on the temperature sensitivity of the agent to be entrapped, this could affect the stability of the agent. GSH, however, was stable during heating to 65°C. Another consideration relates to the predominance of DPPC in the endogenous phospholipid pool of the lung. Because of this apparent compatibility, it is possible that the risk of any toxic effects with DPPC liposomes may be low.

In conclusion, this study demonstrates that liposomes administered to the lung show specific pulmonary recovery patterns and solute release characteristics. The choice of lipid composition appears to be a key determining factor. The judicious selection of a suitable liposome formulation could serve to alter the pharmacokinetics of an entrapped active agent for different pulmonary applications.

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