Pharmaceutical Research, Vol. 7, No. 11, 1990

Report

Nebulization of Liposomes. I. Effects of Lipid Composition

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Received January 3, 1990; accepted April 25, 1990

A series of multilamellar liposome dispersions was prepared from lipids of soy phosphatidylcholine or hydrogenated soy phosphatidylcholine containing from 0 to 30 mol% of either cholesterol, stearylamine, or dipalmitoyl phosphatidylglycerol. The liposome dispersions were aerosolized with a Collison nebulizer for 80 min at an output flow rate of 4.7 liters of air/min. The effects of nebulization on the vesicles were determined by monitoring the release of encapsulated 5,6-carboxyfluorescein (CF) from dispersions containing ≈200 μg of total CF, of which 93.1 ± 2.4% (N = 18) was initially encapsulated. In all experiments CF was released from the liposomes while being aerosolized, and this ranged from a mean of 12.7 ± 3.8 to 60.9 ± 1.9% of the encapsulated CF, depending upon the lipid composition. The lipid concentration in the dispersions did not affect the rate or percentage release of CF over a range of ≈0.5 to 50 mg per nebulized dispersion. If liposomes are to be used as drug carriers in an inhalation aerosol a lipid composition should be employed which will minimize the release of encapsulated drug caused by nebulization.

KEY WORDS: aerosols; carboxyfluorescein; liposomes; multilamellar vesicles; nebulizers.

INTRODUCTION

The concept of modulated drug therapy by inhalation aerosol has been neglected in favor of other approaches to improve upon the percentage of the administered dose which reaches the lung. While some improvement, via metered dose inhaler systems and nebulized systems, has taken place over the last 20 years (1), generally no more than ≈20% of the dose reaches the lung, and the delivered drug quantity depends upon the ability of patients to control their respiration and to use the inhaler system correctly (1,2).

One plausible method of targeting and retaining drug within the lung is through the use of inhaled liposome preparations. Liposomes can be produced in a variety of sizes and forms (4) and stability problems associated with storage have been largely overcome (5,6). Administration directly to the lung would deliver drug to the site of action and therefore avoid the stability problems of intravenous delivery, where liposomes may release their drug content in the blood before reaching the target site (3). Absorption of free drug in solution from the airways to the circulation is generally rapid. By retaining the drug within liposomes, the dose and any toxicity associated with the drug may be reduced. The vesicles themselves would not be expected to pose a toxicity problem, as they are prepared from compounds closely related to those occurring naturally within the lung (7).

There have been few publications describing attempts at delivering liposome encapsulated drugs to the lung. McCullough and Juliano (8,9) noted that by administering liposome encapsulated cytosine arabinoside by intratracheal installation, the drug was retained in the lung for an extended period, whereas the free drug was absorbed rapidly. Farr *et al.* (10) investigated the possibility of delivering liposomes by nebulizer by tagging the phospholipids with 99^m technetium and following the deposition and clearance of the radiolabel. This was successfully followed up recently (11), where sodium cromoglycate encapsulated in liposomes was administered by nebulizer to the airways of human volunteers and the resulting pharmacokinetic profiles were compared with those of the free drug. The liposome encapsulated drug was retained in the lung for more than 24 hr. Other attempts, by aerosol or instillation, have been with pentamidine (12), enviroxime (13,14) oxytocin (15), benzylpenicillin (15), metaproterenol (16), and superoxide dismutase (17). With the exception of the work with pentamidine, where the authors found that 75 ± 16% of the drug remained encapsulated after aerosolization (12), it has been accepted that the process of nebulization has no effect on the retention of drug within the liposomes. If the drug were to leak rapidly from liposomes while undergiong aerosolization, then the effectiveness of such a preparation, whether for modulated systemic absorption or extended local action, would be reduced or even lost completely. This paper describes the effects of nebulization upon a range of liposomes carrying a neutral, net negative, or net positive charge and demonstrates that nebulization can have a marked effect on the retention of a water-soluble compound within liposomes.

MATERIALS AND METHODS

Preparation of Liposomes. Liposome components were soybean phosphatidylcholine (SPC) (Phospholipon 90,

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0724-8741/90/1100-1127$06.00/0

American Lecithin Co., Atlanta, GA), hydrogenated soybean phosphatidylcholine (HSPC) (Epikuron 200H, Lucas Meyer Inc., Decatur, IL), dipalmitoyl phosphatidylglycerol (DPPG) (Avanti Polar Lipids, Birmingham, AL), cholesterol (CH) (99+% GC grade, Sigma Chemical Co., St. Louis, MO), and stearylamine (SA) (Sigma Chemical Co., St. Louis, MO). Carboxyfluorescein (CF) [5(6)-carboxyfluorescein; Kodak Eastman, Rochester, NY] was used as a water-soluble model "drug" and was purified according to the method of Ralston *et al.* (18). Liposomes were prepared by an extrusion technique similar to the method of Olson *et al.* (19). Briefly, 75 mg of total lipid was dissolved in chloroform or a chloroform/methanol mixture (70:30 by volume) and added to a round-bottom flask containing ≈50 glass beads. The solvent was removed using a rotary evaporator (Büchi Rotavapor, Büchi Laboratoriums Teknik, Flawil, Switzerland), under vacuum, above the transition temperature of the combined lipids for 0.5 hr. The thin lipid film was then further dried for 2 hr using a vacuum pump (W. H. Curtin & Co., Fort Wayne, TX). After drying, 10 g of a heated (65°C) buffer solution containing 15 mg/ml purified CF was added, with an osmolality of ≈300 mosmol/kg. The resultant dispersion was gently shaken for ≥2 hr at 60–65°C and then extruded a total of 3× through 1.0-μm polycarbonate membrane filters (Nucleopore Inc., Pleasanton, CA). The dispersion was finally dialyzed at 4°C against phosphate-buffered saline (PBS) of equivalent osmolality to that of the CF solution. Frequent changes of the external buffer were made over a period of ≥2 days to remove the free CF. The stock preparations were refrigerated at 4°C in glass, under nitrogen, until used.

Characterization of Liposomes. This was carried out by transmission electron microscopy (TEM) and by laser light-scattering techniques. For TEM, samples of stock lipid dispersion were diluted 1 in 10 using PBS buffer. To 100 μl of the suspension 100 μl of 2% (w/v) phosphatungstic acid (PTA) in PBS buffer was added and mixed for 1 min. A single drop was placed on a Formvar and carbon-coated 400-mesh copper grid (EMSL Supplies, Westmont, NJ), which was then drawn off using filter paper until the surface of the grid was almost dry. A drop of distilled water was added to the grid and allowed to stand for 10 sec before being drawn off completely. The dried sample was observed under the electron microscope (Phillips 301, Phillips Instruments Inc., Mahwah, NJ) using a 60-kV beam. For sizing vesicles by laser light scattering, 20-μl samples of the dialyzed dispersions were placed into cylindrical glass holders and diluted with 0.5 ml of PBS buffer filtered through a 0.1-μm membrane filter (Millipore Corp., Bedford, MA). The sample was then sealed and mixed thoroughly before being placed in the sample holder of a laser particle sizer (Nicomp Model 270; Particle Sizing Systems Inc., Goleta, CA). Samples were run until a stable size reading was obtained.

Carboxyfluorescein and Lipid Content of Liposomes. Stock samples of 100 μl in triplicate were removed and diluted 5000× in PBS buffer. The diluted dispersion was alkalinized with 50 μl of 2 *M* NaOH per 5 ml to ensure that the CF was completely ionized and the sample was analyzed for free CF content by spectrofluorimetry (Perkin-Elmer LS-3 fluorescence spectrometer; Perkin-Elmer Corp., Norwalk, CT) at the excitation and emission wavelengths of 490 and 515 nm, respectively. Samples of 5 ml were retained and a further 50 μl of 10% (w/v) Triton X-100 was added to all samples except those containing cholesterol. The samples were mixed thoroughly and allowed to incubate at room temperature for ≥10 min before a reading for the total CF was taken. Liposomes containing cholesterol did not release all CF at any concentration of Triton X-100 which was employed (20,21). Consequently, for these liposomes three further 100-μl samples were removed from the stock preparation to determine the total CF. These were diluted with 100% methanol (Fisher HPLC grade; Fisher Scientific, Springfield, NJ), which was found to release all the encapsulated CF. Results for the CF content of the tested liposome preparations are shown in Table I. Calibration curves were prepared in PBS and in methanol. The slopes obtained from the calibration curves in the aqueous medium were consistently ≈88% of the slopes obtained from the methanol calibration curves, indicating that the quantum yield for CF in PBS is less than in methanol. Lipid concentrations were determined by first extracting the lipids according to the method of Bligh *et al.* (22) and then performing the phosphate assay "for large spots" described by Rouser *et al.* (23). Values for the lipid content were obtained from the phosphate data using a lipid:phosphate ratio of 8.3.

Nebulization of Liposomes. The nebulizer system is shown in Fig. 1. A Collison nebulizer (24) (3 inlet/outlet; BGI Inc., Waltham, MA) was used to produce a fine aerosol spray with droplets of micron dimensions. Compressed air was supplied to the nebulizer by a clinically used air pump (M/S pump; Medical Specifics, Dallas, TX) at a flow rate of 4.7 liters/min and 15.6 psig as measured by a Bourdon gauge. Samples of the stock liposome dispersions were diluted to 50 g with PBS such that the total CF content was 200 μg/50 g (Table I). The dispersions were nebulized for a total of 80 min and samples of 100 μl were removed at set intervals from the dispersion in the glass Collison flask. These samples were analyzed for free (C_{free}) and total (C_{tot}) CF concentrations in the nebulized dispersion as described above. In the case of cholesterol containing liposomes 2 × 50-μl samples were removed, one of which was analyzed for free CF by dilution in PBS and the other analyed for total CF by dilution with methanol. Using the above methods it is assumed throughout that the expression

$$C_{tot} = C_{free} + C_{encaps} \quad (1)$$

is valid with (C_{encaps}) representing the increase in concentration (μg/ml) which would occur within the dispersion after bursting open the liposomes with detergent or methanol. In practical terms only the free and total concentrations of CF are determined and the encapsulated concentration is found by subtraction. However, for comparison purposes it is convenient to express the release of CF from the liposomes as a percentage of the CF (% release) which was encapsulated prior to nebulization. Thus

$$\% \text{ release} = \frac{[C_{free} - C_{free(0)}]}{[C_{tot} - C_{free(0)}]} * 100 \quad (2)$$

with $[C_{free(0)}]$ representing the concentration of free CF in the disperson before nebulization. Since it was convenient to use 200 μg of CF/50 g of PBS as a starting value for each

Table 1. Characteristics of Prepared Liposomes

Liposome composition[a]		Content of CF (μg/ml)[b] Free	Encapsulated	% of total CF encapsulated[c] Initial	Final	Release	Lipid[d] content (mg/ml)
SPC	A	30.4	335.8	91.7	63.7	28.0	4.58
	B	20.2	131.4	86.5	53.4	33.1	2.25
SPC:DPPG 9:1	A	18.0	210.1	92.1	43.6	48.5	2.62
	B	31.8	556.2	94.6	37.2	57.4	4.10
SPC:DPPG 7:3	A	49.4	555.2	91.1	43.0	48.1	2.97
	B	25.3	506.2	95.0	46.4	48.6	4.42
SPC:CH 9:1	A	21.5	357.7	94.0	43.1	50.9	4.57
	B	33.7	387.5	91.3	41.5	49.8	4.66
SPC:CH 7:3	A	18.9	266.3	92.9	80.0	12.9	4.22
	B	13.0	122.8	89.4	64.3	25.1	3.47
SPC:SA 9:1		9.3	175.2	94.7	74.1	20.6	2.74
HSPC		31.6	671.3	95.3	52.9	42.4	3.03
HSPC:DPPG 9:1		23.5	534.8	95.6	56.4	39.2	5.02
HSPC:DPPG 7:3		24.8	468.6	94.7	52.7	42.0	3.07
HSPC:CH 9:1		31.0	534.8	94.2	35.1	59.1	3.50
HSPC:CH 7:3		18.2	283.9	93.6	74.5	19.1	3.11
HSPC:SA 9:1		18.3	294.6	93.8	70.9	22.9	3.77

[a] SPC = soy phosphatidylcholine (787); HSPC = hydrogenated soy phosphatidylcholine (791); DPPG = dipalmitoyl phosphatidylglycerol (735); CH = cholesterol (387); SA = stearylamine (270). Mole ratios are based upon the stated molecular weights in brackets. The letters (A) and (B) refer to individual batches of the stated composition.

[b] Content is expressed as μg of CF per ml of stock liposome preparation. 'Free' refers to the content of CF (μg/ml) in the buffer solution after dialysis and 'encapsulated' refers to the content of CF within the liposomes (μg/ml). Results are the average of 3 experiments.

[c] The percent of the total CF which was encapsulated per unit volume of dispersion before starting an experiment (initial), the remaining percent encapsulation after a nebulization period of 80 min (final), and the percent release over this period. Results are the average of 3 experiments.

[d] Lipid content of the stock liposome dispersions as determined from phosphate assay (Ref. 23).

experiment, the quantity of lipid associated with this amount of dye varied for each preparation tested (Table I). To ensure that the lipid concentration was not affecting the CF release from the liposomes, nebulization was carried out with HSPC liposomes containing 10 mol% of DPPG at lipid concentrations of ≈0.5, 2.5, 5, 25, and 50 mg/50 g PBS. Duplicate experiments were performed for each lipid concentration tested.

The aerosol droplet size and size distribution were determined by collecting the aerosol in an Anderson Mark II cascade impactor (Anderson Samplers Inc., Atlanta, GA). The deposited droplets were washed off each of the stages using 0.5 ml of a 0.5% (w/v) Triton X-100 solution and wiping the plate surfaces clean with a tissue wipe. The final stage contained a depth filter and it and the tissue wipes were placed within individual 20-ml vials and weighed. Vials were diluted with a 0.5% (w/v) Triton X-100 solution and 100 μl of 2 *M* NaOH and reweighed. Solutions were mixed and then sonicated (Branson ultrasonic cleaner, Branson Ultrasonics Inc., Danbury, CT) for ≥5 min to ensure release of all CF from the liposomes on the tissues. The aqueous samples were finally centrifuged at 10,000 rpm (J2-21 centrifuge, Beckman Instruments Inc., Palo Alto, CA) and the supernatant was decanted and directly assayed for the presence of CF by fluorimetry.

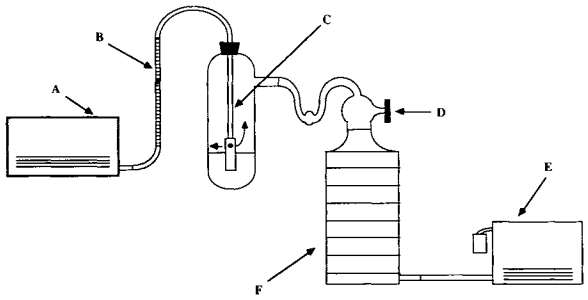

Fig. 1. Schematic of the nebulizer system. (A) Compressed air pump (4.7 L/Min outflow); (B) air flow meter; (C) Collison nebulizer; (D) direction of diluent air flow—through filter to mixing chamber and then to the cascade impactor; (E) vacuum pump (28 L/min inflow); (F) Anderson mark II nonviable cascade impactor.

RESULTS AND DISCUSSION

Characterization of Liposomes. Typical electron micrographs of liposomes are shown in Fig. 2. It was not possible to differentiate between liposomes of different compositions apart from qualitatively noting some aggregation of vesicles not carrying a net positive or negative charge. It is clear that a range of vesicle sizes exists below or around the 1-μm pore diameter of polycarbonate membranes through which the vesicles were extruded. The multilamellar nature of the liposomes is also apparent, although the bilayers often appear misshapen because of the negative staining process. Good results were obtained with laser light scattering for the liposomes containing DPPG. The size of the liposomes was seen to be stable over at least 2 months. Stable readings were difficult to obtain with the liposomes of other compositions again because of aggregation and subsequent sedimentation during data collection by the laser particle sizer. Settling was

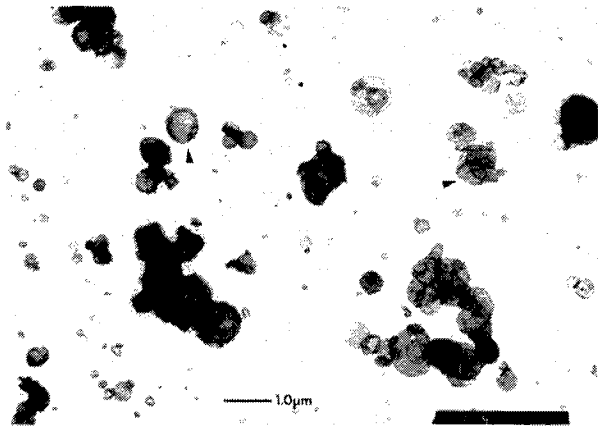

Fig. 2. Negative stain electron micrograph of HSPC:DPPG liposomes (9:1 mole ratio) extruded through 1.0-μm polycarbonate filters. Heterogeneity of size is evident but the vesicles remain at or below ≈1.0 μm. The multilamellar structure is indicated by the arrowheads.

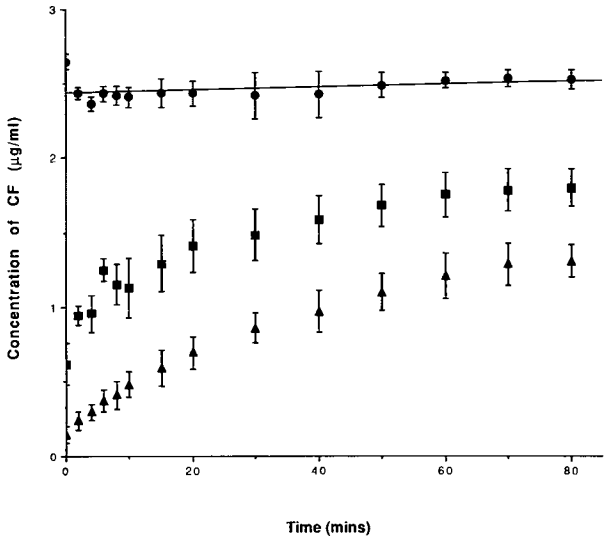

Fig. 3. Effect of methanol vs Triton X-100 at releasing CF from nebulized SPC:CH 9:1 mole ratio liposomes. The concentration increase in CF through nebulization alone is shown as the lower plot (▲). A small increase in concentration occurs through the addition of detergent, producing a final concentration of 0.25% (v/v) Triton X-100 (■). Incubation of a separate sample with methanol demonstrates the complete release of the encapsulated CF (●).

also noted for the neutral liposomes on storage. Nonetheless, all liposomes were readily dispersed before use and <10% of encapsulated CF was leaked from any of the liposome preparations, some of which had been stored for over 3 months.

Carboxyfluorescein Content of Liposomes. The content of CF in each of the stock liposome dispersions as micrograms per milliliter is given in Table I. Although only a final concentration of 0.25% (v/v) Triton X-100 was present in diluted samples removed from the nebulized dispersions, this was quite sufficient to release the CF from non-cholesterol-containing liposomes. The percentage of CF assayed from liposomes disrupted with detergent and incubated for ≥10 min was 100.8 ± 4.4% (N = 8) of liposomes treated with methanol. This was not the case for the liposome preparations containing cholesterol, which were resistant to the solubilizing effects of the detergent. Under the same conditions release of entrapped CF was variable, ranging from 15.1 to 94.6% that obtained in methanol. The problem that can arise if Triton X-100 is used alone is illustrated in Fig. 3, where liposomes of SPC and 10 mol% of cholesterol were nebulized for 80 min. The 100-μl samples were first diluted to 4 ml and assayed for free CF. The samples were retained and 100 μl of Triton X-100 was added and mixed and then read again. A relatively constant increase in the CF concentration is noted which is not representative of the total CF present in the nebulized dispersions. Separate samples, diluted similarly with methanol, illustrate the release of all CF and show the nearly constant level of total CF present in the samples during the nebulization period. The nature of the solute will also affect the conditions required to release it from liposomes, and therefore care should be taken to determine that all solute is released from the liposomes when conducting similar studies.

Nebulization of Liposomes. For all liposome compositions tested there was a release of encapsulated CF to the surrounding buffer solution during nebulization and the lipid composition of the vesicles markedly affected their ability to resist leakage of CF while being nebulized. After an 80-min testing period the percentage release of encapsulated CF was smallest with HSPC liposomes containing 30 mol% of cholesterol (12.7% ± a range of 3.8%, N = 3) and greatest with liposomes of SPC and 30 mol% of DPPG (60.9 ± 1.9%, N = 3). Control experiments performed with the nebulizer switched off showed no release of CF to the buffer over the 80-min testing period.

The presence of the DPPG had little influence on the release of CF from HSPC liposomes (Fig. 4). In contrast, the

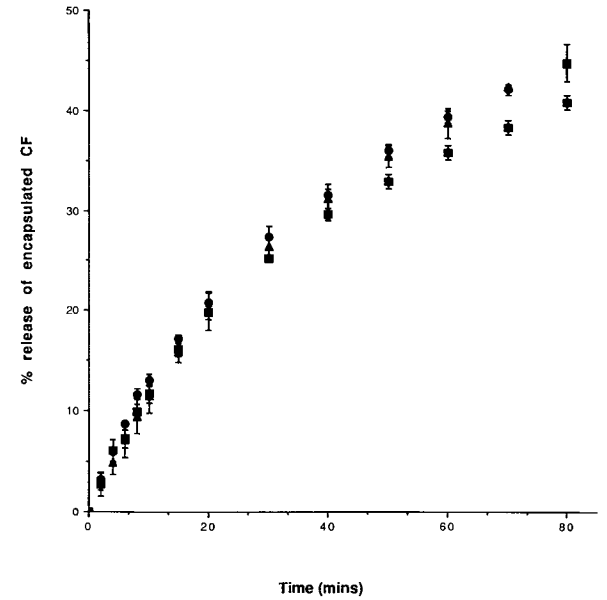

Fig. 4. The percentage release of encapsulated CF from HSPC liposomes containing 30 mol% DPPG (▲), 10 mol% DPPG (■), and no DPPG (●) during an 80-min period of nebulization. Results are the average of three experiments for each lipid composition. Error bars represent the range.

impact of DPPG on the unsaturated SPC was quite marked (Fig. 5). DPPG at a 10 or 30 mol% composition caused an increase in the release of the CF over plain SPC liposomes. In fact, two independent batches of SPC:DPPG liposomes were tested (A and B, Table I) and similar results were obtained, although some batch-to-batch variation did occur, implying that factors other than the lipid composition were also influencing results.

Cholesterol is known to stabilize liposomes and reduce their permeability to solutes (25). However, liposomes of both HSPC and SPC containing 10 mol% CH resulted in a marked increase in the release of encapsulated CF over their HSPC or SPC counterparts (Figs. 6 and 7), while liposomes containing 30 mol% CH showed the opposite effect (Figs. 6 and 7). The results imply that liposomes containing 10 mol% CH have a looser packing arrangement than liposomes without CH but the opposite is true of vesicles containing 30 mol% CH. The results may be corroborated by X-ray studies conducted by Johnson (26), who has noted that 10 mol% cholesterol causes an increase in the surface area of liposomes and a decrease in bilayer thickness but further increases in cholesterol content increased the bilayer thickness without changing the surface area.

The presence of SA in both the SPC and the HSPC liposomes reduced the release of CF relative to the plain liposomes (Fig. 8). Unfortunately it was not possible to prepare liposomes containing 30 mol% of SA for further comparison, as the "liposomes" appeared to aggregate during preparation and could not be extruded through the polycarbonate filters.

The overall results may in part be determined by the influence of the DPPG, CH, and SA on the molecular packing of the lipid bilayers. The DPPG is, like HSPC, a saturated lipid and in the gel state at room temperature. Therefore its influence on the molecular packing of the HSPC might be small: keeping the bilayers well packed together despite the repulsive effects of the negative charge, whereas the presence of the saturated fatty acid chains of the DPPG as well as the net negative charge in the bilayers of the SPC

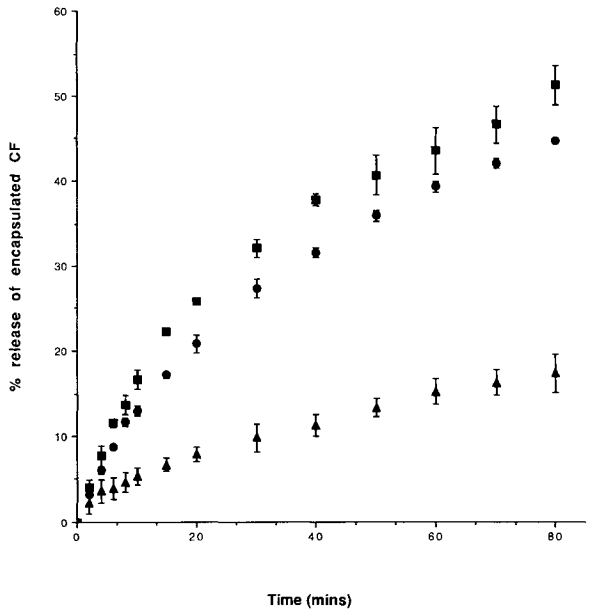

Fig. 6. The percentage release of encapsulated CF from the HSPC liposomes containing 30 mol% CH (▲), 10 mol% CH (■), and no CH (●) during an 80-min period of nebulization. Results are the average of three experiments for each lipid composition. Error bars represent the range.

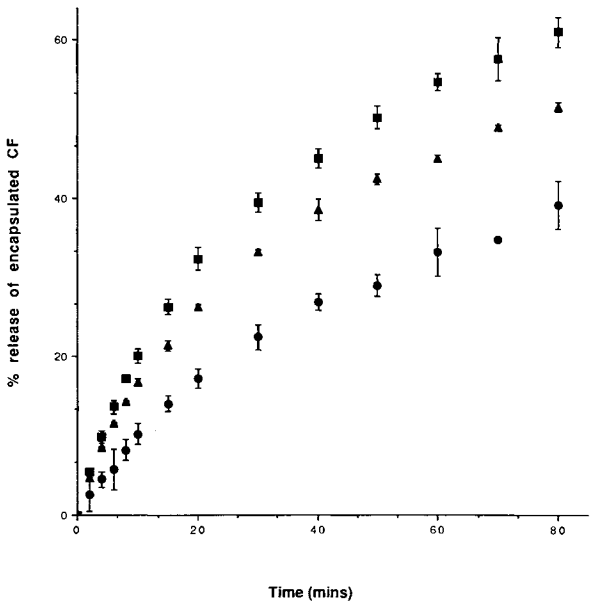

Fig. 5. The percentage release of encapsulated CF from SPC liposomes containing 30 mol% DPPG (▲), 10 mol% DPPG (■), and no DPPG (●) during an 80-min period of nebulization. Results are the average of three experiments for each lipid composition. Error bars represent the range. For the purposes of clarity, only one set of data is shown for each of the compositions (set b, Table I).

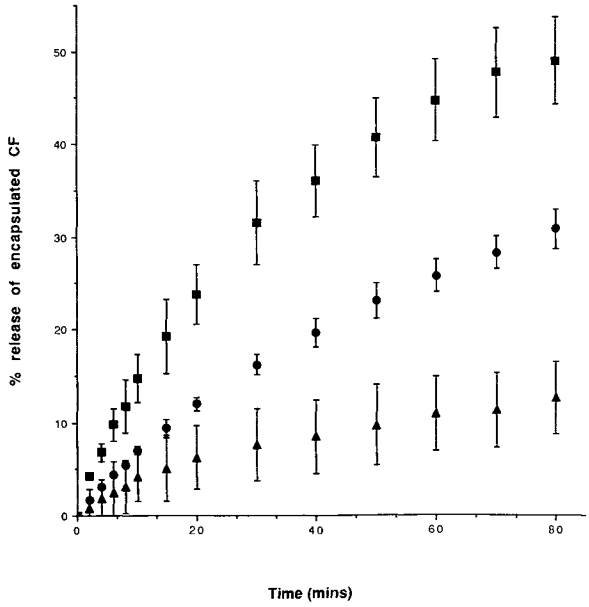

Fig. 7. The percentage release of encapsulated CF from SPC liposomes containing 30 mol% CH (▲), 10 mol% CH (■), and no CH (●) during an 80-min period of nebulization. Results are the average of three experiments for each lipid composition. Error bars represent the range. For the purposes of clarity, only one set of data is shown for each of the compositions (set a, Table I).

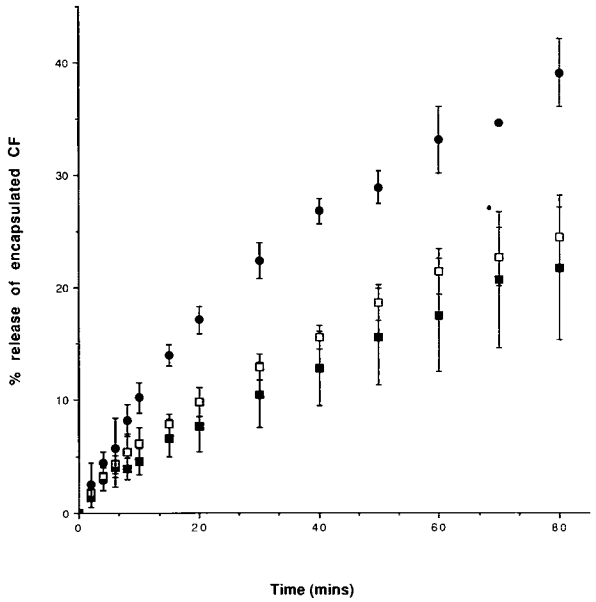

Fig. 8. The percentage release of encapsulated CF from SPC (■) and HSPC (□) liposomes containing 10 mol% SA during an 80-min period of nebulization. Results are the average of three experiments for each lipid composition. The release of CF from SPC (●) liposomes (set b, Table I) is shown for comparison.

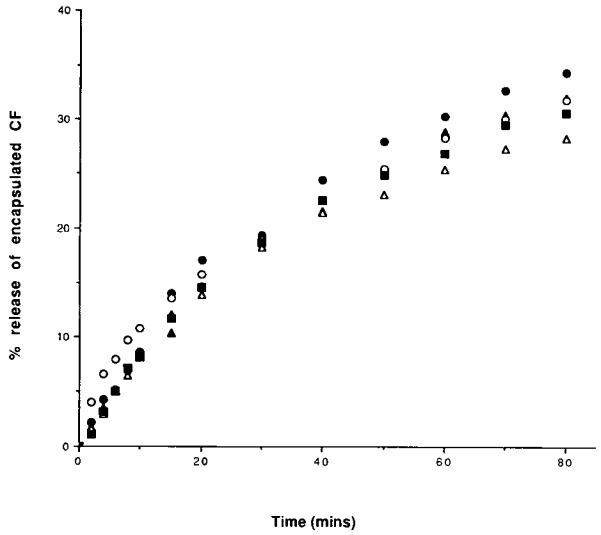

Fig. 9. The average percentage release of encapsulated CF from liposome dispersions with a lipid content of ≈0.5 mg (○), 2.5 mg (△), 5 mg (▲), 25 mg (■), or 50 mg (●) lipid/50 g of dispersion in PBS. No obvious difference in CF release characteristics occurs over the range of lipid concentrations used. Each plot is the average of two experiments.

might cause the mixture of the two lipids to arrange in a less dense packing configuration. Possible phase separation may also occur through the mixing of the gel like DPPG with the liquid crystalline state of the SPC (27).

The stearylamine presumably has the ability to intercalate readily in both the SPC and the HSPC liposomes since it has only a single hydrocarbon chain and a "tighter" packing may result thereby minimizing the effects of nebulization. However, stearylamine containing liposomes have been shown to have a much higher toxicity to cells *in vitro* (28) than the other lipid components and therefore it is unlikely that it would be used in any potential liposome product.

No obvious difference was observed in the CF release profiles for any of the nebulized lipid concentrations between ≈0.5 and 50 mg/50 g dispersion (Fig. 9). This indicates that the variable CF release from the liposomes of the different compositions was not a function of the lipid concentration in the dispersions.

The aerosol droplet size and not the size of the liposomes dictated the size distribution found by cascade impaction (Fig. 10). The mass median aerodynamic diameter was 1.2 μm ± 1.7 σ_g (N = 10). Although the liposomes themselves were extruded through 1.0-μm filters, the results have been confirmed, from data not presented here, for liposomes of a range of sizes. This has also been observed by Farr *et al.* (10), where the droplet size was found to determine the pulmonary deposition of radiolabeled liposomes in the lungs of human volunteers.

The results demonstrate that the process of nebulization can cause the release of solute encapsulated within liposomes. If a drug–liposome formulation is prepared for the purpose of inhalation by aerosolization, it should be demonstrated that there is minimal release of the drug from the liposomes during nebulization. Likewise, where a liposome inhalation preparation is to be compared against an inhaled solution of the free drug, as has already been performed (11–14), the above consideration should also be borne in mind. By employing a lipid composition which minimizes solute release, a step toward an optimal liposome inhalation preparation can be achieved. Other factors influencing solute retention include the vesicle size, the operating characteristics of the nebulizer system, and the physicochemical characteristics of the drug in question. Studies investigating these factors are currently under way. It can be expected

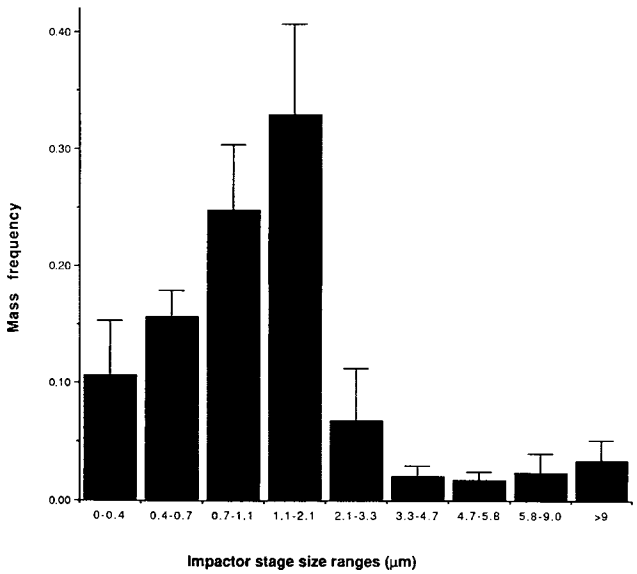

Fig. 10. The frequency distribution of assayed CF found on each stage of the cascade impactor. The size ranges for each of the stages reading from left to right are 0–0.4, 0.4–1.1, 1.1–2.1, 2.1–3.3, 3.3–4.7, 4.7–5.8, 5.8–9.0, and greater than 9.0 μm. Error bars are the standard deviation (N = 10).

that liposome characteristics will require to be tailored for a drug in order to retain it in the vesicles during nebulization.

ACKNOWLEDGMENTS

The invaluable assistance of Donna Williams and company of the ICBR electron microscopy core facility of the University of Florida is gratefully acknowledged, together with the wonderful glass work fabricated by Rudy Strohschein of Southern Scientific Inc. The Epikuron 200H was a gift from Stan Barnes of Lucas Meyer Inc. This work was supported by NIH Grant AI 26339-01A1 and a grant from the Florida High Technology and Industry Council.

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