Nebulization of Liposomes. III. The Effects of Operating Conditions and Local Environment

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Multilamellar liposomes (MLV) of saturated phosphatidylcholine and dipalmitoyl phosphatidylglycerol (DPPG) (9:1 mole ratio) containing 5,6-carboxyfluorescein (CF) were prepared and extruded through 1.0-μm polycarbonate membranes. Diluted aqueous dispersions were aerosolized for a total of 80 min using a Collison nebulizer under a variety of conditions. The effects of air pressure, temperature, buffer osmotic strength, and pH on nebulized liposome dispersions were studied. Changes in air pressure produced large changes in the percentage release of CF and ranged from 1.3% (4 psig) to 88.2% (50 psig) after 80 min of nebulization. The temperature of the nebulizer dispersions dropped during experiments. The extent of the temperature drop varied according to the air pressure used and ranged from 5°C (4 psig) to 11°C (≥30 psig). The temperature of dispersions caused no increase in CF release until the gel-to-liquid crystalline transition temperature was exceeded (54.6°C), whereupon a 20% increase in leakage was observed after 80 min of nebulization. Aerosol mass output was relatively unaffected by the starting temperature of experiments when conducted within the ambient temperature range. Leakage from the liposomes was increased in hypotonic solution but decreased in hypertonic solutions. At a buffer pH of 2.85 the percentage leakage of CF was increased ≈18% compared to that at pH 7.2 and pH 10.75. Results show that the stability of liposomes composed of saturated phosphatidylcholine and DPPG (9:1 mole ratio) is affected by the operating and environmental conditions under which aerosolization takes place, with air pressure having the greatest effect.

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

INTRODUCTION

The impact of nebulization on liposome stability has been examined with respect to the lipid composition and size of liposomes (1,2). These previous studies were performed using fixed operating conditions where the liposomes were affected by the process of nebulization but were not otherwise adversely affected by the local environment into which they were placed. Because a fixed air pressure was forced through the nebulizer to create the aerosol, the energy supplied to the nebulizer over time was constant. It is reasonable to expect that if the air pressure is varied, the degree of damage to liposomes will also vary. Similarly, the environment into which the liposomes are placed such as the temperature and the pH and osmotic strength of the buffer medium may also affect their stability. Although it is unlikely that nebulization will be conducted under extreme conditions where being used in inhalation therapy, it is important to recognize to what extent the local environment can be altered before unacceptable damage to liposomes composed of commonly used lipids, will occur.

In this study the effects of nebulizer jet air pressure, buffer pH, buffer osmotic strength, and temperature on the stability of liposomes during nebulization are examined. The influence of nebulizer fluid temperature on aerosol mass output and the effect of air pressure on the temperature of the nebulizer fluid are also assessed.

MATERIALS AND METHODS

Liposome Preparation

Liposomes of hydrogenated soy phosphatidylcholine (HSPC) (Epikuron 200H, Lucas Meyer Inc., Decatur, IL) and dipalmitoyl phosphatidylglycerol (DPPG) (Avanti Polar Lipids Inc., Alabaster, AL) were prepared as described previously in a ratio of 9:1 HSPC:DPPG (1). The experiments studying pH used liposomes composed of distearoyl phosphatidylcholine (DSPC) (Avanti Polar Lipids Inc.) and DPPG at the same ratio. The solute encapsulated in the liposomes was 5(6)-carboxyfluorescein (Kodak Eastman, Rochester, NY), which was purified as described previously (1). All liposome preparations were extruded three times through polycarbonate filters of 1.0-μm pore size prior to dialysis (1). A method of calculating the individual weights of lipids required to obtain a desired mole ratio in a predetermined total quantity of lipid is described in the Appendix.

Liposome Nebulization

Nebulization with a stainless-steel three-jet Collison nebulizer (BGI Inc., Waltham, MA) was used in all experiments except those where pH was studied. In these experiments the nebulizer body was constructed from Delron acetal resin plastic (E. I. Dupont de Nemours and Co. Inc., Wilmington, DE) according to the specifications detailed by May (3). A volume of liposome stock dispersion equivalent to 200-μg total dye content was placed in a glass Collison flask and diluted to 50 g with the appropriate medium unless otherwise stated below. Nebulization was carried out for a total of 80 min.

Liposomes and Pressure

Air pressures ranging from 4 to 50 psig were used. The liposomes were diluted in isotonic phosphate-buffered saline (PBS) at ≈280 mosmol/kg. The composition of the buffer was 8 g NaCl, 0.91 g KCl, 0.14 g Na_2HPO_4, and 0.19 g KH_2PO_4 per liter of distilled water. Air was supplied from a compressed air tank with a two-stage regulator attached. Samples were removed periodically from the nebulized dispersions and assayed for their content of free CF and total CF by fluorimetry (Perkin-Elmer LS-3 fluorescence spectrometer; Perkin-Elmer Corp., Norwalk, CT) (1). The change in temperature of the nebulizer dispersions was also

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recorded during the experiments by using a digital thermometer (Fisher Scientific, Orlando, FL) combined with a flexible temperature probe which was placed in the nebulizer dispersion close to the body of the nebulizer. Finally, outlet air flow rates associated with each pressure were determined by attaching a calibrated rotameter (Model 10A 10–27A, Fisher and Porter Inc. Warminster, PA) to the outlet of the nebulizer flask.

Liposomes and Temperature

A fixed pressure of 15.6 psig produced by an electric compressor (M/S pump, Medical Specifics, Dallas, TX) was used to drive the nebulizer. The PBS buffer was heated to one of a range of temperatures between room temperature and 63°C and then added to the Collison nebulizer flask prior to the addition of the liposome stock dispersion. The decrease in temperature from the addition of the liposome stock was negligible. Immediately after adding the liposome dispersion to the heated buffer solution, the total dispersion weight was determined (≈50 g) and it was then placed in a water bath thermostated to the required temperature before starting the experiments. The time between the addition of the liposomes to the buffer and the start of experiments was never greater than 2 min. As temperature may also affect the stability of liposomes, control experiments, without nebulization, were conducted at the different starting temperatures. The temperature of the gel-to-liquid crystalline phase change of the HSPC:DPPG 9:1 liposome compositions was determined using differential scanning calorimetry (DSC) (Perkin Elmer Model 7, Perkin Elmer Corp., Norwalk, CT). A 30 μl sample of the liposome stock solution containing ≈5 mg lipid/ml was sealed in an aluminum pan. A sample of 30 μl PBS was used as the reference. A scan was made between 40 and 60°C at a rate of 5°C/min.

Liposomes and Osmotic Strength

The solutions used with the liposomes are described in Table I. The final osmolalities of the solutions were determined experimentally using a vapor pressure osmometer (Model 5000, Wescor, Logan, UT). Control experiments in water, 0.05 *M* NaCl, PBS, and 1.0 *M* NaCl were performed without nebulization.

Liposomes and pH

The DSPC:DPPG 9:1 liposomes were nebulized in 50 g of PBS buffer solution with the pH adjusted to 2.85 (0.01 *M* HCl), 7.21, or 10.75 (0.01 *M* NaOH). Stock buffer solutions of 500 ml were prepared and 12 *M* HCl or NaOH was added to give final concentrations of 0.01 *M* HCl or NaOH in the solutions. The final pH values and osmolalities are given in Table I. Buffers were used within 4 weeks of preparation. For these experiments, the samples were analyzed in a rapid, convenient fashion which has not previously been described. Two samples of 50 μl were removed at each time point during nebulization and were placed directly into wells of a microtiter plate (96-well cell culture cluster dish; Costar, Cambridge, MA). Each well contained 50 μl of PBS. In the case of the acid experiments this buffer solution was adjusted with NaOH to ensure that the final pH would be greater than 9. Once all samples were collected, the plates were "read" for the unquenched fluorescence using a fluorometric microtiter plate reader (Cytofluor 2300 fluorescent measurement system, Millipore Corp., Bedford, MA) fitted with bandpass filters of 485-nm excitation and 530-nm emission wavelengths. Liposomes were then disrupted by the addition of 110 μl of a 1:1 methanol:tertiary butyl alcohol mixture (Sigma Chemical Co., St. Louis, MO) to each of the plate wells. The final solutions were then well mixed before being reanalyzed by the plate reader to obtain values for the total unquenched fluorescence. Calibration curves were obtained for both the aqueous and the aqueous–organic mixture. Control experiments were also performed at the same pH's without nebulization.

RESULTS AND DISCUSSION

Liposomes and Pressure

Pressure has a major influence on the leakage of CF from liposomes during nebulization (Fig. 1). At 4 psig (2.4 L/min air flow) 1.3% CF was released, whereas at 50 psig (11.1 L/min air flow) 88.2% CF released over a period of 80 min. The relationship between the air flow rate and the air pressure is described by

$$\text{air flow (L/min)} = 0.19 \times \text{air pressure (psig)} + 1.63 \quad (1)$$

The relationship between the percentage release and the air pressure is approximately linear during the early stages of the experiments but becomes increasingly nonlinear as they progress (Fig. 2). There are two possible reasons for these observations. (a) The liposomes contain a quantity of CF which is not readily released when damaged during nebulization at any of the air pressures employed (2). How quickly the "accessible" CF is released will depend upon the energy transfer to the liposomes from the air flow forced through the nebulizer jet. From the samples taken during the initial

Table I. Characteristics of Solutions Used to Study the Effects of pH and Osmolarity on Liposomes

Solution[a]	pH	Osmolality	Solution[b]	Osmolality
0.01 *M* HCl	2.75	275	Water	8
PBS	7.21	269	0.05 *M*	95
0.01 *M* NaOH	10.75	269	PBS	269
			0.25 *M*	588
			0.5 *M*	822
			1.0 *M*	1611

[a] The 0.01 *M* HCl and NaOH were the final concentrations of the acid and alkali after addition to PBS.
[b] The molar concentrations of NaCl in distilled water except where otherwise stated.

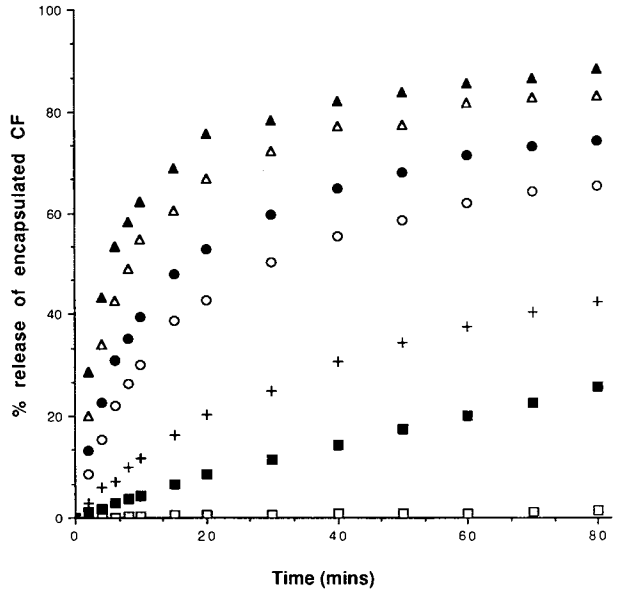

Fig. 1. The mean percentage release of encapsulated CF from liposomes of HSPC:DPPG 9:1 during nebulization at a variety of air pressures. The air pressures studied were 4 (□), 10 (■), 20 (○), 30 (●), 40 (△), and 50 (▲) psig. Results are also plotted for experiments performed at 15.6 psig generated by an air compressor (+). Data shown are the mean of duplicate experiments at each pressure.

stages of nebulization, there may have been insufficient time to release much of the accessible CF at any air pressure, resulting in linear plots in Fig. 2 at the early time points. The less "accessible" CF requires a longer period of nebulization before its release becomes apparent at the higher air pressures toward the latter stages of the experiments. The curvilinear nature of the plots obtained from the later time points might therefore be explained. (b) Despite the extrusion through 1.0-μm polycarbonate membranes, the liposomes will retain a size distribution below this upper 1.0-μm limit. The effects of size on the leakage of CF has already been demonstrated (2). As the size of the liposomes is reduced, so is the leakage of CF. At the start of nebulization the larger liposomes will be damaged to a greater extent than the smaller ones. Toward the end of the nebulization when the large liposomes have released much of their CF, the release from the smaller liposomes may become apparent. However, the accumulation of free CF will be slower because they are less affected by the nebulization process and contain less CF than their larger counterparts. Again, the rate at which the CF is released from the liposomes will depend on the energy supplied from the air pressure used. Hence the apparent linear release with air pressure at early time points when the large liposomes are still being damaged and the nonlinear release at later time points when the energy input from the high pressure has released much of the CF from the large liposomes and not the smaller ones.

The impact of air pressure has important practical considerations. Too high a pressure will rapidly damage the liposomes and perhaps render the treatment ineffective. Too low a pressure will require a prolonged period of nebulization in order to deliver a sufficient dose. This will be inconvenient to both the patient and staff who may be required to administer the medication.

As the air pressure was increased the cooling of the nebulizer dispersion was increased. However, the temperature drop of the dispersion did not increase at air pressures greater than 30 psig. The time to reach the equilibrium temperature was approximately 40 min for all the air pressures used (Fig. 3).

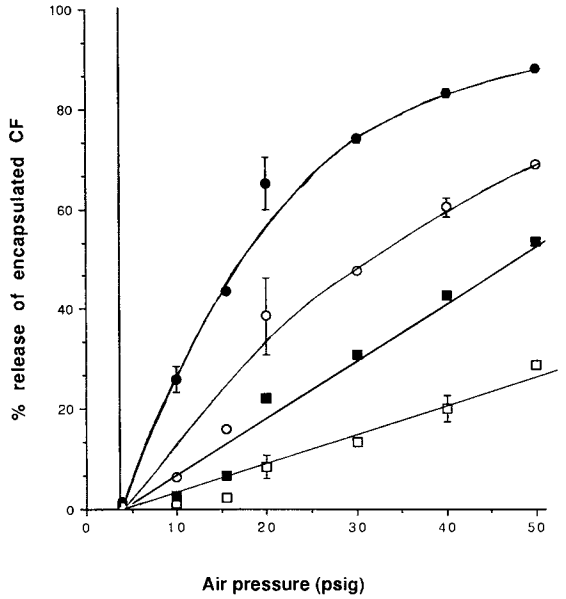

Fig. 2. The mean percentage release of CF from liposomes as a function of pressure after 2 (□), 8 (■), 15 (○), and 80 (●) min of nebulization. Data shown are the mean of two experiments. The error bars are the range. The lines indicate the linear and curvilinear nature of the plotted data.

Liposomes and Temperature

Temperature does not affect the release of CF from the liposomes until they are nebulized above the transition tem-

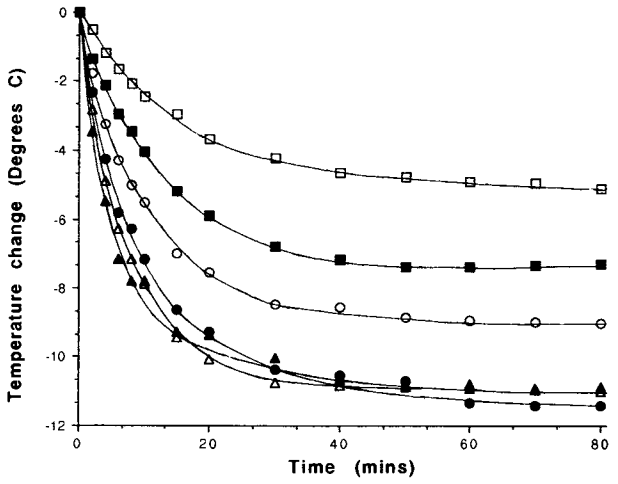

Fig. 3. The mean temperature drop of liposome dispersions with an initial mass of 50 g during nebulization conducted at different air pressures: 4 (—□—), 10 (—■—), 20 (—○—), 30 (—●—), 40 (—△—), and 50 (—▲—) psig. After approximately 40 min the steady-state temperature was reached at all pressures studied. Data shown are the mean of duplicate experiments at each pressure.

perature of 54.6°C as determined by DSC (Fig. 4). In early experiments, liposomes nebulized at a starting temperature of exactly 60°C showed little difference in CF release compared to results obtained at lower temperatures. This was due to the equilibrium temperature of the dispersion being at or below the transition state temperature of the liposomes. When maintained above the temperature of the phase change, a substantial increase in the percentage release of CF was observed. Control experiments without nebulization demonstrated no increase in release of CF until also maintained at a level above the transition temperature, where ≈20% of the encapsulated CF was released after 80 min (Fig. 4). By subtracting this temperature data from the data combining temperature and nebulization effects, the resulting percentage release plot is similar to the plots obtained at lower temperatures where no effect due to temperature was observed (Fig. 4). This implies that the increased leakage of CF above the transition temperature is due to the temperature alone and is independent of the leakage induced by nebulization.

In experiments which were started at ambient temperature the total mass loss as well as the temperature decrease was recorded over the 80-min period of nebulization. A recent article by Kongerud *et al.* (4) describes a close linear relationship between the mass output of a Wright nebulizer and the variations in room temperature. To see if a similar relationship existed using the liposome dispersions in a Collison nebulizer, the mass outputs for 81 experiments started at room temperature and a fixed air pressure of 15.6 were plotted against the equilibrium temperature of the solutions. A positive but poor correlation (R^2 = 0.23) was found (Fig. 5). It is logical to expect increased aerosol output at higher temperatures due to an increase in loss of water vapor. However, if the water vapor pressure above the dispersions can be considered indicative of the escaping tendency of the solvent, the small differences in water vapor pressure which will exist above solutions in the starting temperature range of 22 ± 3°C (5) will result in only small changes in mass loss, which will be readily masked by "normal" experimental er-

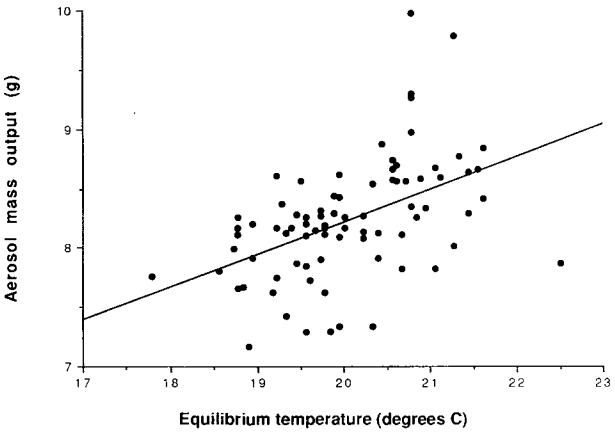

Fig. 5. The relationship between the total aerosol mass output and the equilibrium temperature of the liposome dispersions during nebulization.

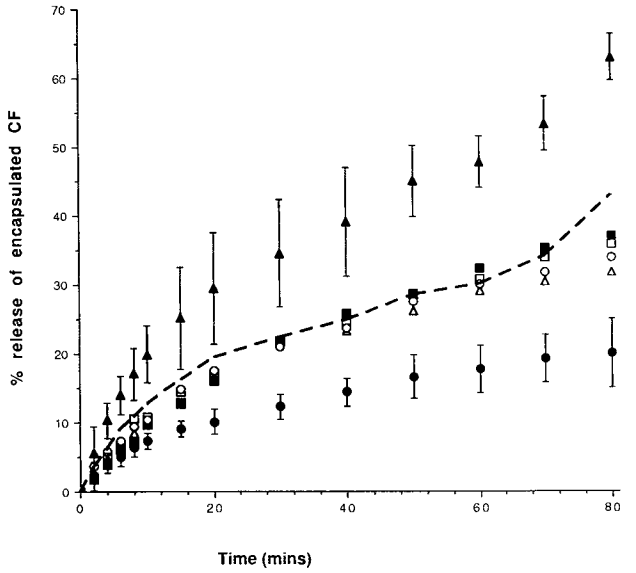

Fig. 4. The effect of temperature on the mean percentage release of CF from liposomes during nebulization. The starting temperatures were 22°C (■), 32°C (△), 37°C (□), 45°C (○), and >60°C (▲). An increase in percentage release of CF occurred when the steady-state temperature was maintained above that of the transition temperature (54.6°C) of the lipid composition of the liposomes. Some release of CF occurred during control experiments conducted at 60°C (●). When the percentage release of CF due to the 60°C controls is subtracted from the equivalent nebulization data, a release profile (——) similar to those performed at below the transition temperature is obtained. Data shown are the mean of ≥3 experiments at each temperature.

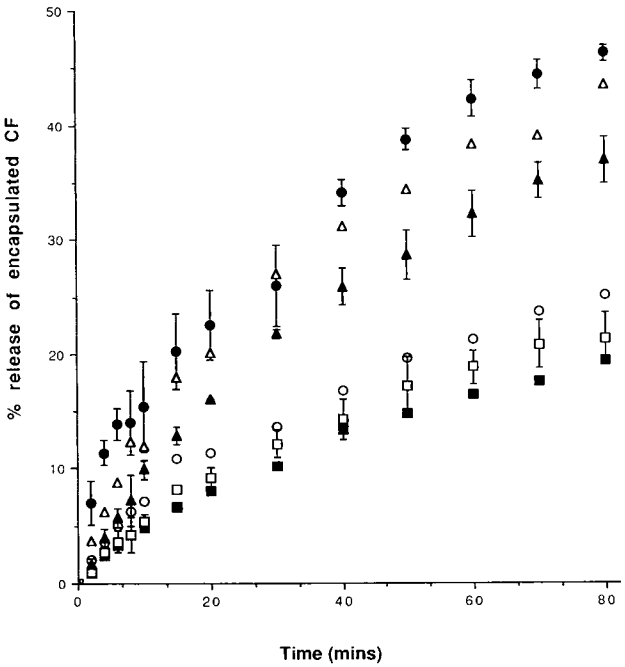

Fig. 6. The effect of osmotic strength on the mean percentage release of CF from liposomes during nebulization. The saline concentrations were 0 (●), 0.05 *M* (△), ≈0.15 *M* (PBS; ▲), 0.25 *M* (○), 0.5 *M* (■), and 1.0 *M* (□). Data shown are the mean of three experiments at each salt concentration. Error bars, where shown, are the range.

ror. It is therefore unlikely that a distinct linear correlation between ambient temperature and output will be noticed.

Liposomes and Osmotic Strength

When liposomes were nebulized in water and in 50 m*M* NaCl there was a noticeable increase in the release of CF from the liposomes compared with liposomes dispersed in solutions of higher osmolality (Fig. 6). Similarly, there was some reduction in leakage of CF from the liposomes when nebulized in hypertonic solutions. This effect is presumably due to the liposomes swelling in the solutions of low saline concentration and shrinking in the higher concentrations (6). In control experiments there was no release of CF from the liposomes dispersed in isotonic or hypertonic solutions. However, a linear release of CF occurred from liposomes placed in the hypotonic solutions over an 80-min period (Fig. 7).

Liposomes and pH

No substantial change in CF release from the experiments carried out at physiological pH was observed for pH's of 7.21 and above (Fig. 8). At pH 2.85 an increase was observed. Release of CF from the liposomes during control experiments occurred only in the pH 2.85 buffer, where ≈4% release was observed after 80 min. The increase in release of CF when nebulized at pH 2.85 was ≈18% greater than the total release at pH 7.2. The increase is probably due to an increase in the percentage of nonionized CF within the liposomes at the low pH. Consequently, the nonionized compound will be more capable of partitioning across the lipid bilayers. The agitation of the liposomes during nebulization may enhance the leakage rate of the CF. It can be expected that the pH effect will also be dependent upon the nature of the solute and the lipids. Cationic compounds will presumably follow the opposite trend seen with the CF, while the net charge of the lipids at the pH of the experiment will also influence leakage rates by either repelling, attracting, or even binding the solute.

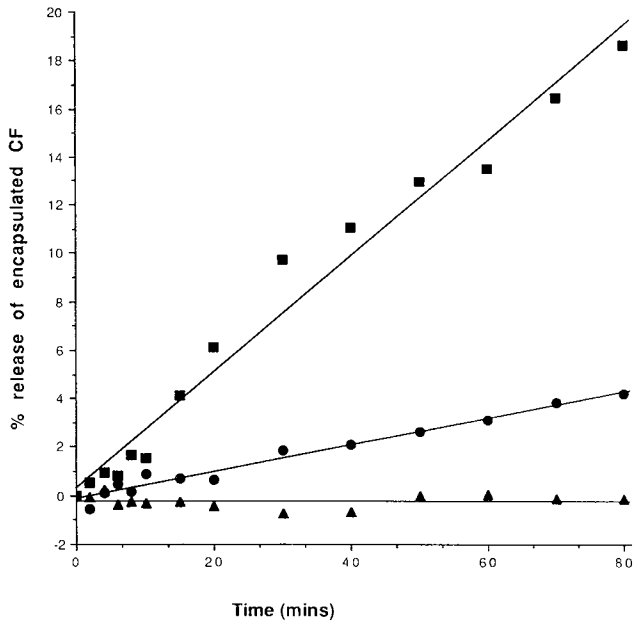

Fig. 7. The effect of osmotic strength alone on the mean percentage release of CF from liposomes. Results are shown for CF release in distilled water (■), 50 m*M* NaCl (●), and ≈0.15 *M* NaCl (PBS; ▲). Lines are the least-squares regression fits of the mean data. The respective osmolalities of the solutions were 8, 95, and 269 mosmol/kg. Data shown are the mean of ≥3 experiments at each temperature.

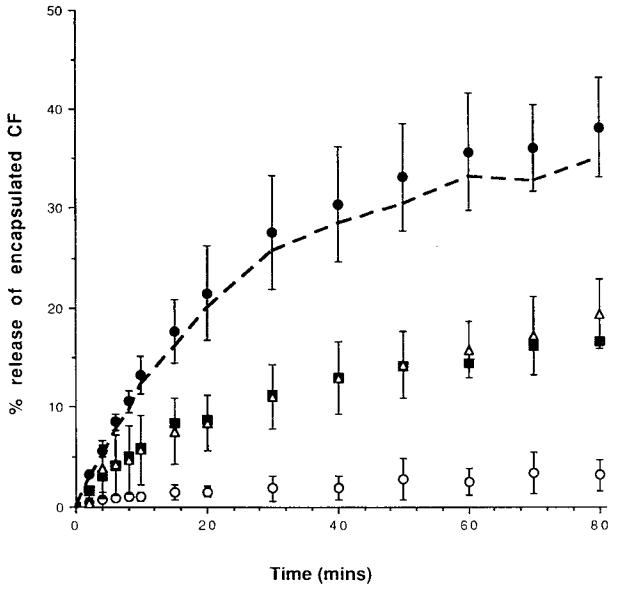

Fig. 8. The effect of pH on the mean percentage release of CF from liposomes during nebulization. The starting pH's were 2.85 (●), 7.21 (△), and 10.75 (■). The pH 2.85 nebulization data minus the percentage release resulting from the control experiments (○) obtained at pH 2.85 are also shown (——). The data shown are the mean of three experiments at each pH. Error bars, where shown, are the range.

The percentage release of the CF from the liposomes composed of DSPC:DPPG 9:1 at physiological pH was noticeably less than for the liposomes composed of HSPC:DPPG 9:1. This is either due to an effect of the slightly different lipids which were used or, more probably, due to the differences in the manufacture of the plastic vs the stainless-steel Collison nebulizer.

In conclusion, to minimize leakage of CF from liposomes of saturated phosphatidylcholine and DPPC at a 9:1 molar ratio the following optimal conditions were established: (a) the pressure should be kept to a minimum; (b) the buffer medium is better if hypertonic; (c) the pH should be high enough to ensure that the CF, or anionic drug, is ionized; and (d) the temperature should not be greater than the gel to liquid crystalline phase transition. The experiments have been restricted to using one type of liposome composed mainly of a saturated lipid. The effects that the operating conditions and environment will have on the stability of liposomes composed of alternative lipids and containing different solutes remain to be evaluated.

APPENDIX

Liposome compositions are often reported as mole ratios and are commonly prepared by the solvent evaporation

technique. The individual weights, n_i, of the lipids required to prepare the correct mole ratio in a lipid mixture needs to be calculated. Given that the total weight of lipids, n_{tot}, required is known, then

$$\sum_{i=1}^{N} n_i = n_{tot} \tag{A1}$$

where N is the number of lipids to be used in the mixture. The mole ratio, x_i, of each lipid component is related to the number of moles of the lipid, M_i, by

$$x_i = \frac{M_i}{\sum_{i=1}^{N} M_i} \tag{A2}$$

The number of moles of lipid is in turn related to the weight of lipid required by

$$M_i = \frac{n_i}{MW_i} \tag{A3}$$

with MW_i the molecular weight of the lipid. Substituting the RHS of Eq. (A3) for M_i in Eq. (A2) and rearranging in terms of n_i gives

$$n_i = x_i * MW_i * \sum_{i=1}^{N} \frac{n_i}{MW_i} \tag{A4}$$

which, together with Eq. (A1), provides a series of $N + 1$ simultaneous equations from which the n_i lipid weights can be determined.

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