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# STABILITY OF PHOSPHOINOSITIDES CONTAINING LIPOSOMES: EFFECTS OF TRITON X-100, TEMPERATURE AND 68 RPM

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**ABSTRACT**

The stability of phosphoinositide containing liposomes was investigated. It was found that 2% of triton X-100 completely released entrapped carboxylluorescein. The release of carboxyfluorescein occurred the most of 58°C (78% at 2 hours) and the least at 4°C (5.4% at 118 hours). When stored at 68 RPM (at 25°C) about the 20% carboxyfluorescein was released at 48 hours, but when the liposomes were kept at 25°C for the same period of time, the release was about 10%.

## Introduction

Liposomes consisting of a mixture of phosphoinositides from bovine brain and egg lecithin have been extensively used for the in vitro investigation of the mode of action of aminoglycosides antibitodics such as neomycin (Au et al., 1986). But no study has been made to investigate the stability of such liposomes under various storage conditions.

Carboxyfluorescein (CF), a fluorescent dye, is the most commonly used marker to assess the rates of leakage of water-soluble substances from liposomes. The validity of the data is based on two assumptions that is at higher concentrations (about 100 mM), Cfs fluorescence is self quenched and triton X-100 releases all the dye from liposomes. It was recently shown that the release of CF was dependent on incubation temperature and the detergent concentration (Sila et al., 1986). Therefore in the present study to find the correct amount of dye entrapped within the liposomes, these were incubated with various concentrations of triton X-100 at room temperature. The stability study was made by following the release of CF with the passage of time. The studies were conducted at 68 RMP (an approximate situation of transportation conditions) and at various temperatures.

## Materials and Methods

1-α-phosphatidylcholine from egg yolk (EL), crude phosphoinositide sodium salt mixture (Pl mix; Cat No.P 6023), N- 2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) were purchased from Sigma Chemical Co., (St. Louis, MO, USA).

Carboxyfluorescein (99.4%) and d-a-tocopherol (99%) were from Eastman Kodak, Rochester, NY, USA). Sepharose 2B was from Pharmacia Fine Chemicals (Uppsala, Sweden). The buffer for all experiments was 0.05M HEPES (pH 7.5 and the ionic strength adjusted to 0.2 with sodium chloride). The salts were of A.R. grade.

### *Preparation of Liposomes*

The liposomes contained EL and PI mix in the ratio of 4:1 and the lipid concentration was 12.5 umole/ml. The liposomes also contained one mole % d-α-tocopherol. A thin film of lipids in 20ml scintillation vial was formed under nitrogen while vial was rotated by hand and residual solvent was removed by storing the flask for at least 2 hours under vacuum from water pump. The dried film was resuspended in the HEPES buffer containing 90mM carboxvfluorescein. The resultant multilamellar liposomes were passed through Extruder$^{R}$ (Lipex Membrane Inc., Vancouver, Canada) ten times through a stalk of two 100nm polycarbonate filters (Nucleopore Pleasanton, CA, USA) employing nitrogen pressures upto 250 psi. Free carboxyfluorescein from liposomes was removed by the passage of the dispersion through a 0.8 x 11.3 cm column of Sepharose 2B and the removal of free dye resulted in about seven folds dilution of liposomes.

### *Characterization of Liposomes*

**a) Freeze fraction electron microscopy**

The liposomes were mixed with 20% glycerol (cryoprotectant). They were frozen in freon 22 and were stored in liquid nitrogen. The frozen samples were placed on to the specimen stage of Balzers BAF 301 unit at -170°C. The chamber was evacuated to approximately 5 x $10^{-6}$ mbar and the stage was warmed to -115°C. The sample was fractured and the fractured surfaces were coated with approximately 0.8nm Pt at an angle of 45° and this was followed by carbon coating of approximately 10 nm at an angle of 90°. The replicas were washed in 50% chlorax bleach solution. After rinsing 3 times in double distilled water, the replicas were mounted on 400 mesh copper grids and were examined with a Philips EM 400 transmission electron microscope at a magnification of 178,000-298,000. The photographed areas were selected at random avoiding overlap of the areas.

**b) Photon correlation spectroscopy**

Particle size determination was done by Langley-Ford LSA-2 spectrometer. Light source was 5mW neon laser with an exiting wavelength of 632.8 nm at a fixed scattering angle of 90°. Five ul of samples were mixed with 3 ml of filtered (through 200 tint nitrocelluse membrane filter) deionized water and were examined at 25°C using a sample time of 3 x $10^{-5}$ seconds. The calculations of effective particle diameter were done by Langely-Ford (model 1096 CM64) autocorrelator using64 channels.

**c) $P^{31}$-FT NMR**

About 0.5 ml of heavy water was added to about 1.5 ml of liposomes. The measurements were made at 145.7 MHz with broad band proton decoupling (Bruker WM360 NMR spectrometer) employing a 10mm internal diameter NMR tube. Spectra were accumulated from 4323 scans by employing a 45° pulse (18us) and acquisition time of 0.819 seconds (relaxation delay time = zero seconds) and sweep width of 20 kHz. The spectra were obtained before and after the addition of 60u1 of 100 mM maganese chloride. The areas under peaks were determined by cutting and weighing the peaks.

The number of bilayers in liposomes was calculated by the following formula (]ousma et al., 1987).

$$\text{Number of bilayers} = 100/(2 \times \text{RLOS})$$

where RLOS is the % relative loss of NMR signals in the presence of maganese ions.

**Fluorescent measurement**

All fluorescent measurements were made (at excitation 490nm and emission at 520nm) with a Perkin Elmer LS-5 spectrofluorimeter (excitation and emission slits were 10mm and 3mm respectively). Two microliters of liposomes were added to the HEPES buffer so that total volume of the solution is 3ml. The % release of carboxytlourescein was found by the following formula.

$$\% \text{ Release} = 100 \times (Ft - Fo) / (Fa - Fo)$$

where Ft is the flurescence intensity at t time, Fo fluorescence intensity at zero time and Fa is the flourescence reading after two hours when liposomes were incubated in HEPES buffer containing 4% triton X-100.

**Stability studies**

i) Liposomes were kept at 25°C in HEPES buffer containing 1, 2 and 4% triton X-100 for two hours.

ii) Liposomes were kept at 68 revolutions per minutes in Orbit shake. (Lab-Line Instrument, Inc, II. USA, Model No.3520) and at 4,25,37445,58°C for upto 144 hours.

## Results and Discussion

Particle size analysis of the liposomes (immediately after preparation) by photon correlation spectroscopy revealed that their size be within 99-135 nm. Electron micrographs of liposomes (without CF) stored at 4°C revealed that the most of liposomes (about 100nm) are unilamellar but larger liposomes (about 125nm) have sometimes two lipid bilayers. $P^{31}$-NMR study revealed that about 1.1 bilayers are present in the liposomes.

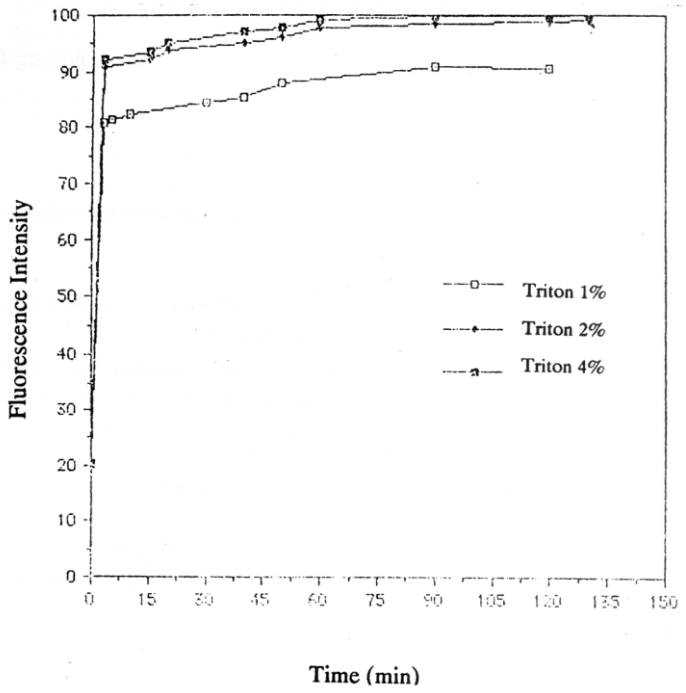


Fig. 1. The effect of Triton X-100 on the stability of liposomes at 25°C.

The effect of triton X-100 concentration on the release of the entrapped CF is shown in Fig.l. It appears that 2 and 4% triton X-100 completely disrupts the liposomes. In a

previous study on multilamellar liposomes, it was found that 5% concentration of triton X-100 was necessary (at room temperature) to release all entrapped CF. However, when the small unilamellar liposomes were incubated with 1 to 5% triton X-100, the release was independent of the detergent concentration (Sila et al., 1986). Thus from $P^{31}$-NMR, freeze fracture electron microscopy, effect of triton X- 100 concentration it can be said that the liposomes used in the present study are not exactly unilamellar.

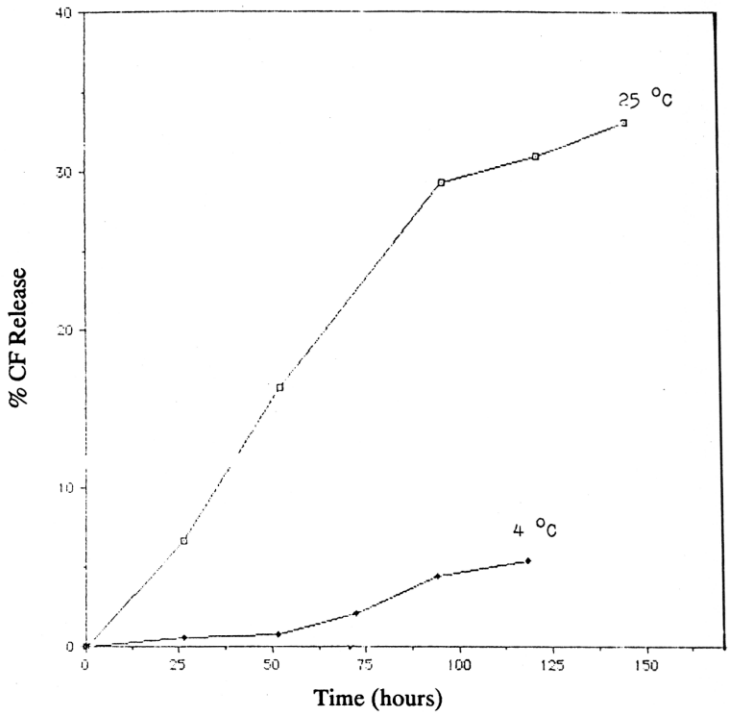


Fig. 2. Stability of liposomes at room temperature and in referigerator.

When liposomes were incubated at various temperatures, 4°C appears to be a right temperature for the storage of liposomes (see Fig. 2, 3). There was a consider-able release of CF at higher temperatures for example the % release of CF was 6.8 and 37 at 45 and 58°C respectively within one hour. The liposomes were kept at 68 RPM (at 25°C in Orbit Shaker), about 20% drug was released within 48 hours but when the liposomes were stored at 25°C for the same period of time, the release was about 10% (Fig. 4). This clearly shows that liposomes lose considerable amount of entrapped substance during transportation.

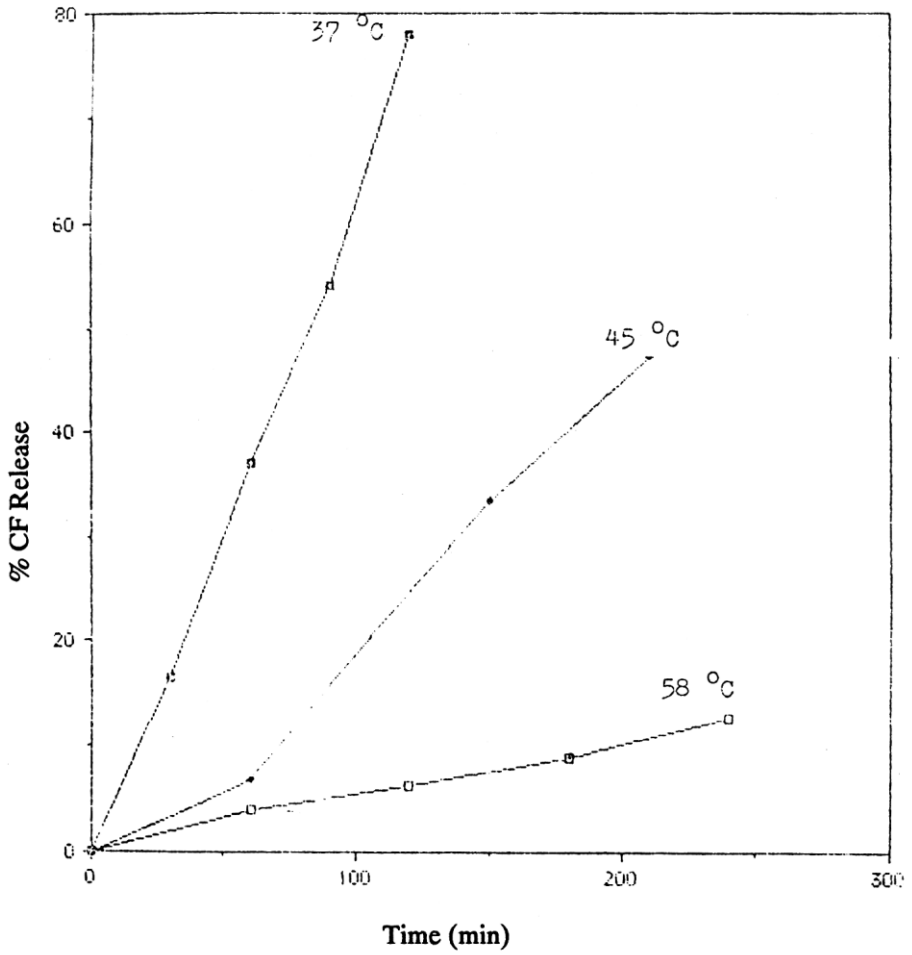


Fig. 3: Liposomes stability at 37, 45, 58°C.

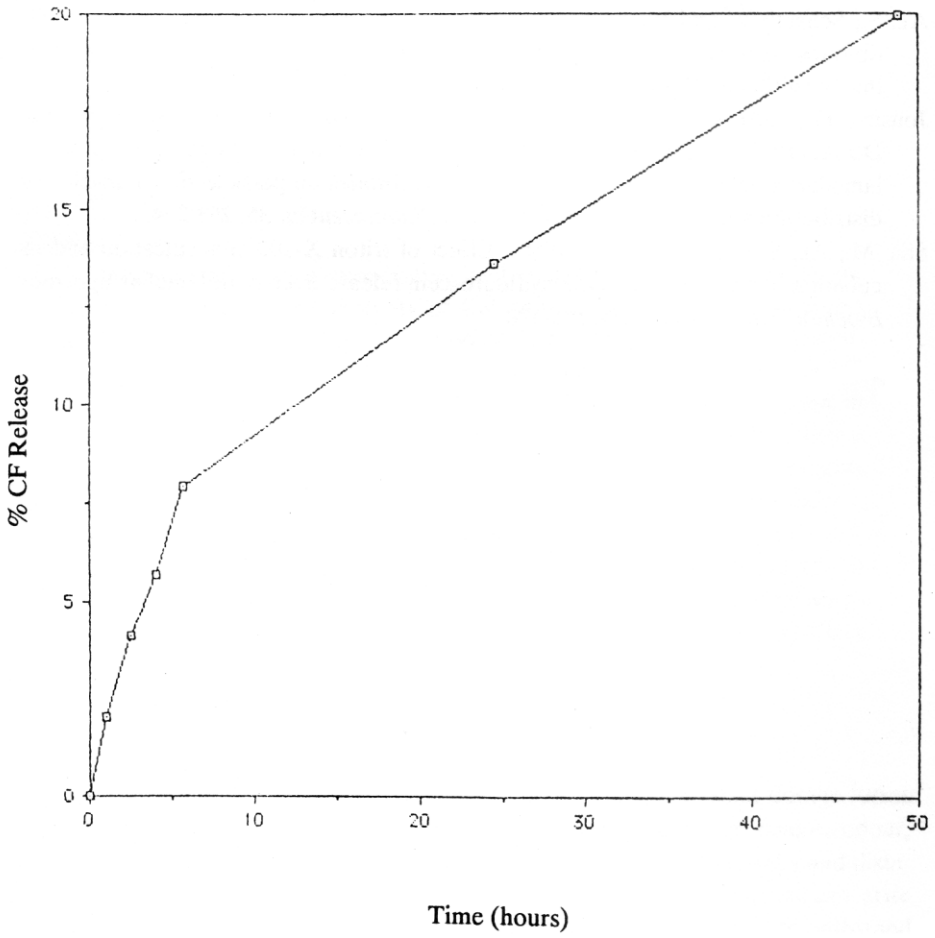


Fig. 4: Stability of liposomes at 68 RPM.

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