INCORPORATION OF HIGH MOLECULAR WEIGHT RNA INTO LARGE ARTIFICIAL LIPID VESICLES

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Summary By utilizing an ether infusion technique with lecithin, cholesterol, and dicetylphosphate, giant liposomes have been produced with diameters ranging from 0.5 to 2.0 microns. These liposomes have been used to sequester 4S, 16S, and 23S E. coli [^{3}H]RNA and can be effectively separated from non-liposome incorporated RNA by ribonuclease treatment followed by Sepharose 4B gel filtration. The [^{3}H]RNA within these liposomes can be extracted and appears to be undegraded.

INTRODUCTION

The ability to incorporate high molecular weight RNA into cells, both in vivo and in vitro, has long been hindered by the failure of previously employed techniques to protect RNA from ribonuclease activity, resulting in the loss of biological function (1). The ability to sequester RNA in artificial lipid vessels (liposomes) would greatly facilitate such studies. Liposomes have previously been employed to entrap a wide spectrum of molecules ranging from small molecular species such as cAMP (2) and 5-fluorouracil (3) to large macromolecules including hexoseaminidase A (4), lysozyme (5) and dextranase (6). Moreover, Magee et al., (7) have shown the incorporation of polyinosinic·polycytidylic acid into both positively and negatively charged liposomes.

The methods utilized for production of liposomes in the above mentioned studies generally yield vesicles, either multi- or unilamellar, approximating 500 A in diameter. These liposomes suffer from the drawback that their aqueous volume is small and trapping efficiency low. Recently, Deamer and Bangham (8) have devised a technique capable of producing large (0.1 to 1 μm) unilamellar liposomes using an ether infusion process. Data presented in this

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paper demonstrates that using the Deamer and Bangham procedure (8), 1) lipid vesicles ranging from 0.1 to 1 μm are produced intact, 2) RNA ranging from 4S-23S is sequestered and 3) entrapped RNA is protected from ribonuclease degradation.

MATERIALS

[^{3}H] Uridine (25.9 Ci/mmole) was purchased from New England Nuclear, Boston, MA. Arsenazo III, dicetylphosphate, and pancreatic deoxyribonuclease I were obtained from Sigma Chemical Company, St. Louis, MO. Pancreatic ribonuclease, Pronase, predigested before use, and hen egg lysozyme were obtained from Calbiochem, San Diego, CA. Egg yolk lecithin, solubilized in chloroform, and cholesterol were provided by Grand Island Biological Company, Grand Island, NY. Pharmacia of Uppsala, Sweden provided Sepharose 4B and G-200 Sephadex.

METHODS

Preparation of E. coli [^{3}H]RNA

The uridine requiring E. coli mutant A115 (U^-) was grown in Hayashi-Spiegelman media (9) containing glycerol as the sole carbon source and 7 μg/ml [^{3}H]uridine. The culture was grown until uridine was exhausted, at which point the bacteria were harvested by centrifugation. The pelleted E. coli were lysed by freeze-thawing in 0.02M Tris, 1 mM magnesium chloride containing 300 μg/ml of lysozyme and 50 μg/ml of deoxyribonuclease. The ruptured bacteria were extracted 3 times with cold water saturated phenol and the RNA precipitated four times in ethanol. Prior to incorporation into liposomes, the RNA was treated with 2 mg/ml of predigested pronase for 1 hour at room temperature in order to eliminate residual ribonuclease activity.

Preparation of liposomes

Liposomes were prepared according to the ether infusion method of Deamer and Bangham (8) with some variations. Briefly, lecithin, dicetylphosphate, and cholesterol, in a 7:2:1 molar ratio, were solubilized in diethylether at a final concentration of 4 μmoles of lipid per ml of ether. (All ether used had been previously treated with iron wire coils in order to reduce the peroxide content). Ten milliliters of the ether solution (40 μmoles of lipid) was placed in a 20ml glass syringe with a teflon plunger and attached to a vertical infusion pump. Either 3 mM arsenazo III (A III) calcium complex prepared according to the method of Weissmann et al., (10) or [^{3}H]uridine labeled E. coli RNA (100 μg/ml) was solubilized in 2 ml of sterile 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (Hepes) pH 7.4 containing NaCl-KCl at a final concentration of 0.145M, and placed in a stoppered Leibig condenser heated to 55° C with a circulating water bath. The ether-lipid phase was injected with an infusion pump into the aqueous solution through a 23 gauge needle which had been inserted through a rubber stopper at the bottom of the condenser. The injection rate was maintained at 0.5 ml/minute. As the ether entered the aqueous phase it evaporated leaving a milky white liposome suspension. All glassware and buffers employed were previously autoclaved.

Evaluation of sequestration of AIII and E. coli [^{3}H]RNA

Following liposome formation, the mixture of liposome-entrapped free [^{3}H] RNA was treated with 20 μg of ribonuclease for 1 hour at room temperature in order to insure maximum separation of the liposome associated and unseques-

Abbreviation used: A III, arsenazo III.

tered RNA on Sepharose 4B gel filtration. Ribonuclease treatment was also employed as a means to demonstrate the true latency of the E. coli RNA. The liposome suspension was applied to a 1.5 x 30 cm Sepharose 4B column previously equilibrated with 5 mM Hepes buffer. Two milliliter fractions were collected and assayed for both the presence of liposomes (light scattering at 650 nm) and [^{3}H]RNA. To control for the nonspecific binding of RNA to the liposomal membrane, E. coli [^{3}H]RNA was added exogenously to preformed empty liposomes under conditions identical to those employed for liposome formation. These liposomes were subsequently treated with ribonuclease and chromatographed on Sepharose 4B.

Sequestration of AIII was determined by filtration of the AIII-liposome suspension through Sephadex G-200 and treatment of the liposome associated dye with 10 mM ethyleneglycol-bis (β-aminoethylether)-N, N'-tetraacetic acid (EGTA) for two hours at room temperature. AIII-calcium has absorption maxima at 620 and 650 nm. Chelation of calcium by EGTA causes a loss in 620 and 650 mn absorption and a concomitant gain in the absorbance at 535 nm (10). Failure to observe an EGTA stimulated drop in the absorbance at 620 nm was taken as proof of AIII latency. The possibility of EGTA in this system being unable to reduce the absorbance of liposomally associated AIII-calcium was controlled for by rupturing the liposomal membrane with Triton X-100 and monitoring the loss in absorbance at 620 mm.

Extraction of liposome-entrapped E. coli [^{3}H]RNA

Liposome containing fractions from Sepharose 4B gel filtration were pooled and incubated for 1 hour at room temperature with 3 mg of predigested Pronase in order to eliminate residual ribonuclease activity. Following Pronase digestion the RNA-liposome suspension (5 ml) was extracted with an equal volume of chilled chloroform-phenol (1:1) containing 2 mg of polyvinylsulfate, 2% w/v Triton X-100, 0.4% w/v sodium dodecyl sulfate, and 600 µg of nonradioactive Pronase treated E. coli RNA. The mixture was agitated and then centrifuged for 10 minutes at 5000 rpm. The aqueous phase was reextracted and finally precipitated 2 times in ethanol.

Analysis of liposomally-entrapped E. coli [^{3}H]RNA

Extracted liposomal RNA was solubilized in 0.5 ml of 5 mM sterile Hepes buffer and applied to a linear 10-40% sucrose gradient prepared in 10 mM sodium acetate buffer pH 5.1, 1 mM EDTA, 100 mM sodium chloride. Untreated E. coli RNA was employed as a standard. The gradients were centrifuged at 25,000 rpm in a SW 41 rotor for 18 hours at 4° C. Following centrifugation the gradient was monitored continuously at 260 nm on an Isco model 640 fractionator. Fractions (0.6 ml) were collected and precipitated with trichoracetic acid onto GS 0.22 µ Millipore filters, dried and counted.

RESULTS

Integrity of the liposomal membrane

The integrity of the liposomal membrane was demonstrated by the use of the Weissmann AIII-calcium-EGTA system (10). AIII containing liposomes were resolved from free dye by Sephadex G-200 gel filtration. By this technique two distinct peaks of 620 nm absorbing material were obtained separated by 30 ml. Peak I eluted in the void volume coincident with a previously run blank liposome standard and had a maximum absorbance of 0.4 at 620 nm.

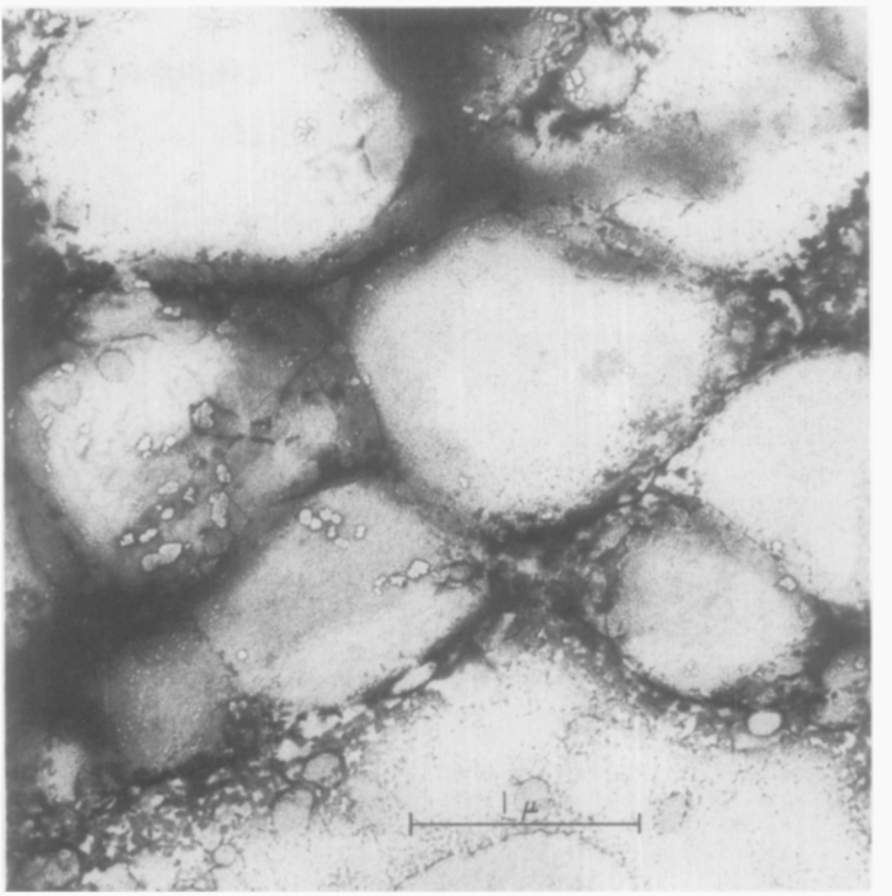

Figure 1. Electron microscopic appearance of liposomes negatively stained with 1% uranyl acetate.

The free dye was recovered in the included volume of the column and had a maximum 620 nm absorbance of 3.0. Pooled peak 1, upon incubation with EGTA, demonstrated no change in absorbance at 620 nm over the 2 hour incubation period. Disruption of the liposomal membrane with Triton X-100 caused an immediate drop in the absorbance at 620 nm from 0.4 to 0.05. Controls with Triton X-100 added to free dye caused only a minimal decrease in the A_{620} reading. An electron micrograph shows the characteristic discrete appearance of liposomes ranging in size from 500A to 2 microns (Fig. 1).

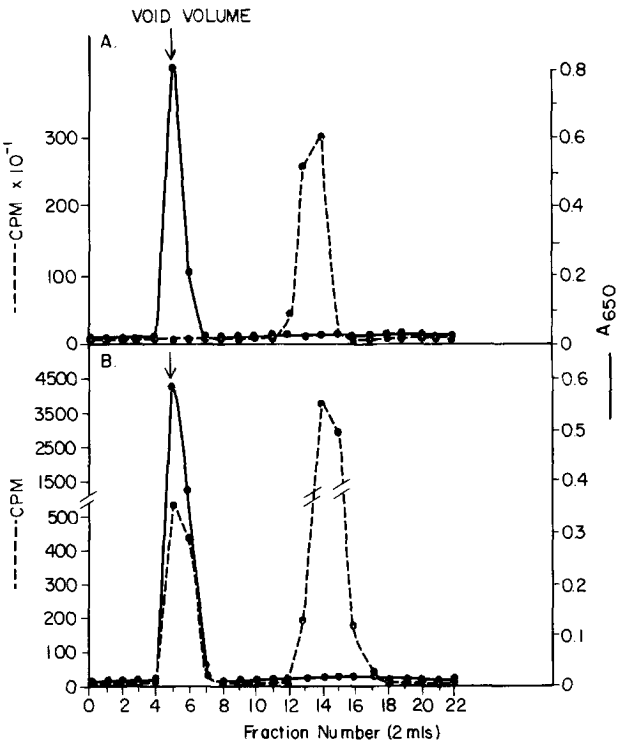

Figure 2. Elution profile derived from a 1.5 X 30 cm Sepharose 4B column equilibrated and run in 5 mM Hepes buffer, 0.145M KCl-NaCl, pH 7.4. (A) Elution pattern obtained using 2 ml of ribonuclease-treated suspension of empty liposomes and exogenously added E. coli [^{3}H] RNA. B) Elution pattern obtained using a ribonuclease-treated 2 ml suspension of E. coli [^{3}H]RNA containing liposomes.

Evaluation of liposome incorporation of E. coli [^{3}H]RNA by chromatographic separation

Ribonuclease-treated [^{3}H]RNA containing liposomes, after application to a Sepharose 4B column, eluted in a sharp A_{650} peak in the column void volume. The [^{3}H]RNA was resolved into 2 fractions, one associated with the liposome peak and one representing free degraded RNA (Fig. 2B). When E. coli [^{3}H]RNA was added exogenously to empty liposomes (see Methods), treated with ribonuclease, and gel filtered, no radioactivity could be found to be associated with the liposome peak (Fig. 2A). This control effectively eliminates the possibility that the liposome associated counts seen in Figure 2B are the result of the nonspecific adherence of [^{3}H]RNA to the liposomal membrane.

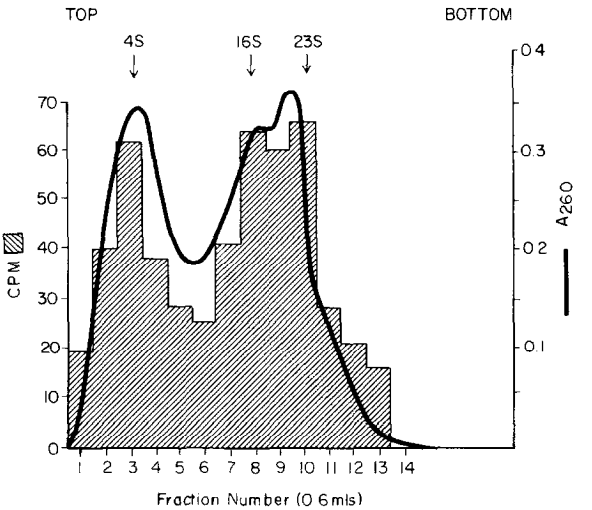

Figure 3. Linear 10-40% sucrose gradient profile of E. coli [^{3}H]RNA extracted from ribonuclease-treated liposomes, (shaded area). Nonradioactive E. coli RNA, added during the first phenol-chloroform extraction, used as a standard (———).

Quantitation of liposomally associated counts varied according to the specific radioactivity of the [^{3}H]RNA employed. All preparations to date have sequestered between 2 and 5 µg of RNA, representing an incorporation of approximately 5% of the added RNA.

Determination of the latency of liposome incorporated E. coli [^{3}H]RNA

Figure 3 represents the sedimentation pattern of liposomally incorporated [^{3}H]RNA on a sucrose gradient. It is evident that this RNA closely parallels the pattern seen for the carrier E. coli RNA. The internal ratio of the liposomal RNA peaks, 4S:16S and 4S:23S, is similar to the corresponding values obtained using the cold carrier RNA. The fact that ribonuclease treatment of the RNA containing liposomes resulted in little if any breakdown of sequestered high molecular weight RNA is taken as conclusive proof of latency.

DISCUSSION

The data presented demonstrates the incorporation of 4S, 16S (MW = 5.6×10^5) and 23S (MW = 1.1×10^6) RNA into large artificial lipid vesicles. The integrity of the vesicles employed and the true sequestration

of high molecular weight RNA has been clearly demonstrated by electron microscopy, chromatographic separation of entrapped from free RNA, and the protection of liposomally incorporated RNA from external ribonuclease digestion, i.e. latency. Moreover, these vesicles also appear to be impermeable to small molecules such as AIII and EGTA. We feel the outlined procedure can now be applied to the liposomal incorporation of various RNA moieties with a wide range of biological activity. Once sequestration has been accomplished, the liposome may serve as a vehicle for the introduction of these macromolecules into eukaryotic cells, both in vivo and in vitro, without the problem of RNA degradation.

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REFERENCES

1. Wang, S. R., Giacomoni, D., and Dray, S. (1973) Exp. Cell Res., 78, 15-24.
2. Papahadjopoulos, D., Poste, G., and Mayhew, E. (1974) Biochim et Biophys. Acta, 363, 404-418.
3. Gregoriadis, G. (1974) Biochem. Soc. Trans., 2, 117-119.
4. Cohen, G. M., Weissmann, G., Hoffstein, S., Awasthi, Y. C., and Srivastava, S. K. (1976) Biochemistry, 15, 452-460.
5. Sessa, G., and Weissmann, G. (1970) J. Biol. Chem., 245, 3295, 3301.
6. Colley, C. M., and Ryman, B. E. (1976) Biochim et Biophys. Acta. 451, 417-425.
7. Magee, W. E., Talcott, M. L., Straub, S. X., and Vriend, C. Y. (1976) Biochim et Biophys. Acta, 451, 610-618.
8. Deamer, D., and Bangham, A. D. (1976) Biochim et Biophys Acta, 443, 629-634.
9. Hayashi, M., and Spiegelman, S. (1961) Proc. Natl. Acad. Sci., 47, 1564-1580.
0. Weissmann, G., Collins, T., Evers, A., and Dunham, P. (1976) Proc. Natl. Acad. Sci., 73, 510-514.