*Biochimica et Biophysica Acta*, 464 (1977) 530–546


BBA 77598

# ENZYME THERAPY VI: COMPARATIVE IN VIVO FATES AND EFFECTS ON LYSOSOMAL INTEGRITY OF ENZYME ENTRAPPED IN NEGATIVELY AND POSITIVELY CHARGED LIPOSOMES

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(Received July 17th, 1976)

## Summary

Entrapment of enzyme in liposomes, biodegradable lipid vesicles, offers an intriguing strategy for the intracellular delivery of these macromolecules to the lysosomal apparatus for enzyme replacement endeavors in selected lysosomal storage diseases. Therefore, the in vivo tissue and subcellular fate and effect on the subcellular distribution of endogenous lysosomal hydrolases was determined following intravenous administration of β-glucuronidase entrapped in positively and negatively charged liposomes into C3H/HeJ β-glucuronidase-deficient mice. Enzyme entrapped in negatively charged liposomes was rapidly cleared from the circulation ($t_{1/2} \approx 4$ min); maximal tissue recovery, 75% of dose, was detected in the liver at 1 h, was maintained for 48 h and then gradually declined to non-detectable levels by 8 days. A similar circulatory clearance and reciprocal hepatic uptake was observed for positively charged liposomes; however, the β-glucuronidase was retained in murine liver for 11 days. Significant activity, 15% of dose, was found in the kidneys up to 1 and 4 days post-injection of positively and negatively charged liposomes, respectively. No activity was recovered in neural or other visceral tissues except in spleen and lungs (<5% of dose). Exogenous β-glucuronidase activity administered in negatively charged liposomes was primarily localized in the lysosomally-enriched hepatic subcellular fraction, compared to the predominantly soluble localization of exogenous activity entrapped in positively charged liposomes. Administration of negatively charged liposomes caused no detectable change in the subcellular localization of several endogenous lysosomal hydrolase activities compared to their distribution in untreated mice. In contrast, a marked but temporary translocation of these hydrolase activities into the soluble fraction was observed following the administration of positively charged liposomes, identifying possible deleterious effects on cellular physiology.

## Introduction

Recent pilot human trials of enzyme replacement have indicated the feasibility of this therapeutic strategy for selected metabolic disorders, particularly the lysosomal storage disorders [1—5]. Reviews of these and earlier replacement endeavors have identified the major barriers to effective therapy [6,8]. Among these obstacles are the short circulating and intracellular half-lives of the exogenous activity, the inadequate delivery to selected target tissue and subcellular sites of pathology, and the inability to monitor the fate and metabolic effectiveness as well as the potential immunological complications of the administered activity. Thus, current efforts have been directed toward the development of techniques to protect the administered enzyme from physiological inactivation and deliver exogenous enzyme to crucial tissue and subcellular sites [9]. In addition, the development of mammalian model systems for the in vivo evaluation of various strategies of enzyme administration prior to human trials has been emphasized [7].

With this perspective, we recently developed a mammalian model system in which the uptake, tissue distribution, and subcellular localization of intravenously administered bovine β-glucuronidase can be directly and sensitively determined in β-glucuronidase-deficient mice [10]. Analogous to the results of pilot human trials, the exogenous enzyme was rapidly cleared from the murine circulation ($t_{1/2} \approx 3$ min), and the majority of administered activity (approx. 70% of dose) was recovered in hepatic lysosomes at 30 min followed by a gradual decline to non-detectable levels by 24 h. In addition, no exogenous activity was detected in all other neural or visceral tissues examined [10]. These findings documented the need to develop protection and delivery techniques designed to minimize the in vivo inactivation and maximize the tissue distribution and retention of the administered activity.

The entrapment of enzymes in microspherules [11], erythrocytes [12], and liposomes [13] has been suggested as a means to protect enzyme from bioinactivation and immunologic surveillance, and/or to enhance enzyme delivery. Chang and Poznansky [14] demonstrated that catalase entrapped in synthetic nylon microspherules was retained in the circulation permitting the effective metabolism of the toxic circulating metabolites in acatalasemic mice. Recently, studies in this laboratory demonstrated that the intravenous administration of β-glucuronidase in autologous erythrocytes prolonged both the enzyme's circulating and lysosomal catalytic half-lives as well as delivered the enzyme to tissues other than liver [15]. Liposomes have been used as vehicles for enzyme delivery in vitro in fibroblasts [16], macrophages [16], HeLa cells [17], and phagocytes [18], and in vivo in rats [16,19—21] and humans [5]. These preliminary studies have indicated that liposomes may be suitable carriers of enzymes for replacement endeavors in selected human diseases. However, the in vivo fates of a mammalian enzyme entrapped in negatively and positively charged liposomes administered intravenously into an enzyme-deficient mammalian model have not been determined. More importantly, the physiological effects of negatively and positively charged liposomes on lysosomal integrity have not been investigated. Therefore, we report here the comparative uptake, tissue distribution and retention, intracellular localization, and effects on lysosomal physiology of

β-glucuronidase entrapped in negatively and positively charged liposomes in β-glucuronidase deficient mice.

## Materials and Methods

*Enzyme preparation.* Bovine liver β-glucuronidase (EC 3.2.1.31; Type B-1, Sigma, St. Louis, Mo.) was further purified about 15-fold by chromatography on Sephadex G-200 (Pharmacia, Piscataway, N.J.) with 0.02 M potassium phosphate buffer (pH 6.5), or the activity was purified from fresh bovine liver by conventional chromatographic techniques [22]. C3H/HeJ *gus*$^h$ male mice (10—14 weeks old, 23—28 g) were purchased from Jackson Laboratories (Bar Harbor, Me.).

*Enzyme assays.* Exogenous bovine (thermolabile) activity was discriminated in murine tissue homogenates from the residual murine (thermostable) activity by the selective thermal inactivation assay of Thorpe et al. [10]. The standard reaction mixture contained 50 μl of enzyme source and 2.33 mM 4-methylumbelliferyl-β-D-glucuronide (Research Products International, Elk Grove Village, Ill.) in 0.05 M sodium acetate buffer (pH 4.6) with 0.1% Triton X-100 in a final volume of 350 μl.

Murine lysosomal hydrolase activities, including total β-hexosaminidase [23] (EC 3.2.1.30), α-galactosidase [24] (EC 3.2.1.22), β-galactosidase [24] (EC 3.2.1.23), β-glucosidase [25] (EC 3.2.1.21), and α-mannosidase [26] (EC 3.2.1.24) were assayed using the appropriate 4-methylumbelliferyl glycoside. Murine arylsulfatase [27] (EC 3.2.1.6.1) was assayed using *p*-nitrocatechol as substrate. One unit of enzymatic activity equaled 1 nmol of substrate cleaved per h in the standard reaction mixtures.

*Liposome preparation.* Negatively charged liposomes were prepared essentially by the method of Gregoriadis et al. [13], with the following modifications. In a 500 ml round bottom flask, 147 mg of dipalmitoyl DL-α-phosphatidylcholine, 22 mg of cholesterol and 20 mg of phosphatidic acid (Sigma) in a molar ratio of 7 : 2 : 1 were dissolved in 25 ml of chloroform/methanol (2 : 1, v/v). For the preparation of positively charged liposomes, an equimolar amount (7.6 mg) of stearylamine (Eastman Organic Chemicals, Rochester, N.Y.) replaced the phosphatidic acid.

A thin lipid film was obtained by rotary evaporation at 37° C and was further dried under a $N_2$ stream for 30 min. Approx. 500 000 units of bovine β-glucuronidase in 15 ml of 3.3 mM potassium phosphate buffer (pH 7.2) was added to the lipid film for entrapment in negatively charged liposomes, whereas enzyme entrapment in positively charged liposomes was carried out at pH 4.0, below the pI (5.1) of bovine β-glucuronidase [28]. For radiolabel experiments, 2 μCi of [1,2-$^3$H]cholesterol (spec. act. 7.4 μg/mCi; New England Nuclear, Boston, Mass.) was added to the lipids and [$^{14}$C]glucose (spec. act. 12.8 μg/μCi, New England Nuclear) was added to the aqueous phase for entrapment in negatively or positively charged liposomes.

Enzymes and/or markers were entrapped in negatively and positively charged liposome preparations by the following procedures. The appropriate lipid film and enzyme solution were dispersed by immersion in a bath sonicator (Ultrasonics Inc., Plainview, N.Y.). The resultant milky suspension was kept at room

temperature for 2 h, and then sonicated at 4°C for 20-s intervals totalling 10 min using a Branson model W185 sonifier equipped with a microprobe (Branson Instruments Co., Stanford, Conn.). The suspension was centrifuged at 100 000 × *g* for 2.5 h in a Beckman L2-65B ultracentrifuge. The supernatant, containing the unentrapped enzyme, was removed and stored for reuse in subsequent liposome preparations. The pelleted positively charged liposomes were then washed extensively by repeated resuspension and centrifugation at 100 000 × *g* for 1 h. The pelleted negatively charged liposomes were resuspended in 4 ml of 6.7 mM potassium phosphate buffer (pH 7.2) with 0.15 M NaCl before passage through a Sepharose 6B column (2.5 × 50 cm, Pharmacia) equilibrated with the same buffer. The turbid fractions of enzyme-loaded liposomes were pooled and centrifuged for 1 h at 100 000 × *g*. The final pellet was resuspended in 4.0 ml of buffer; a 10 μl aliquot was removed, diluted 1 : 100 (to eliminate the inhibitory effect of cholesterol on β-glucuronidase activity [29]), and then assayed for enzymatic activity. The amount of protein entrapped in the liposomes was determined by the method of Kruski and Narayan [30]. A typical liposome preparation contained approx. 6.0 mg protein and 170 mg of mixed lipids in 4 ml of buffer. Liposomes were safely stored up to 1 week at 4°C before use; for all experiments, liposomes were centrifuged to remove any unentrapped enzyme prior to injection. Empty liposomes (negatively and positively charged liposomes) for control experiments were prepared as described above, except no enzyme was present in the loading solution.

*Studies of liposome entrapment.* Following the formation of negatively charged liposomes, the preparation was centrifuged to remove the majority of unentrapped markers, and then chromatographed on Sepharose 6B (flow rate, 0.53 ml/min) to further separate unentrapped from entrapped β-glucuronidase and glucose; [$^3$H]cholesterol incorporation was monitored to determine the association of the entrapped markers with the liposomes. To demonstrate latency of the liposome-entrapped markers, the turbid fractions of negatively charged liposomes were pooled, centrifuged at 100 000 × *g* for 1 h, resuspended in 0.2% (v/v) Triton X-100 in buffer, and incubated at 37°C for 30 min (to insure disruption of the liposome membrane) before reapplication to the Sepharose column. Varying molar ratios of charged lipids were used for the preparation of the lipid film to assess the amount of marker entrapped as a function of increasing liposomal surface charge.

*In vivo studies.* Preparations of bovine β-glucuronidase activity (800—3500 units) entrapped in negatively or positively charged liposomes in a volume of 0.15—0.25 ml of 6.7 mM potassium phosphate buffer (pH 7.2) with 0.15 M NaCl were injected into the tail veins of β-glucuronidase-deficient mice. Mice were killed at desired intervals and blood and organs were immediately removed and prepared for enzymatic assay as previously described [10]. The recovered bovine and endogenous murine β-glucuronidase activities were determined in blood, homogenized organs, and the hepatic subcellular fractions obtained by differential centrifugation [10]. The marker lysosomal hydrolases (β-hexosaminidase, α-galactosidase, β-galactosidase, β-glucosidase, α-mannosidase and arylsulfatase) were assayed in the hepatic subcellular fractions. For tracer experiments, tissue samples were solubilized in 2% dodecyl sulfate for 10 min

at room temperature. Both tissue and blood samples were oxidized with 0.1 ml $H_2O_2$ before addition of 5 ml scintillation solution (Aquasol, New England Nuclear). [$^3$H]Cholesterol and [$^{14}$C]glucose were counted on a Beckman LS-100 scintillation counter with appropriate corrections for quenching and channel overlap.

## Results

*Enzyme and marker entrapment in liposomes*

The following experiments were carried out to demonstrate the incorporation of β-glucuronidase and [$^{14}$C]glucose in negatively and positively charged liposomes. Fig. 1 shows a typical Sepharose 6B chromatographic profile of a radiolabelled negatively charged liposome preparation. The radiolabelled cholesterol and entrapped β-glucuronidase activity and glucose simultaneously eluted in the void volume, whereas unentrapped enzymatic activity and glucose were eluted in subsequent column fractions. Figs. 2a and 2b illustrate the latency of the entrapped markers following treatment of the liposomes with the non-ionic detergent, Triton X-100. When the liposome preparation was

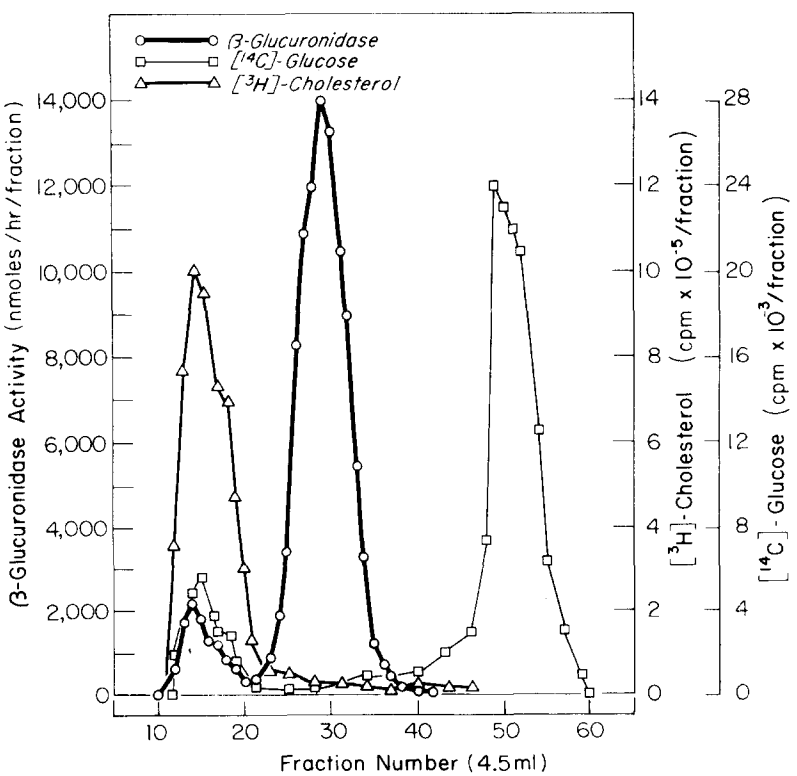


**Fig. 1. Sepharose 6B chromatography of liposome-entrapped and unentrapped markers. Bovine β-glucuronidase and [$^{14}$C]glucose were entrapped in [$^3$H]cholesterol-containing negatively charged liposomes (phosphatidylcholine/cholesterol/phosphatidic acid, 7 : 2 : 1); the preparation was then chromatographed and the fractions assayed for enzymatic activity and radioactivity as described in the text.**

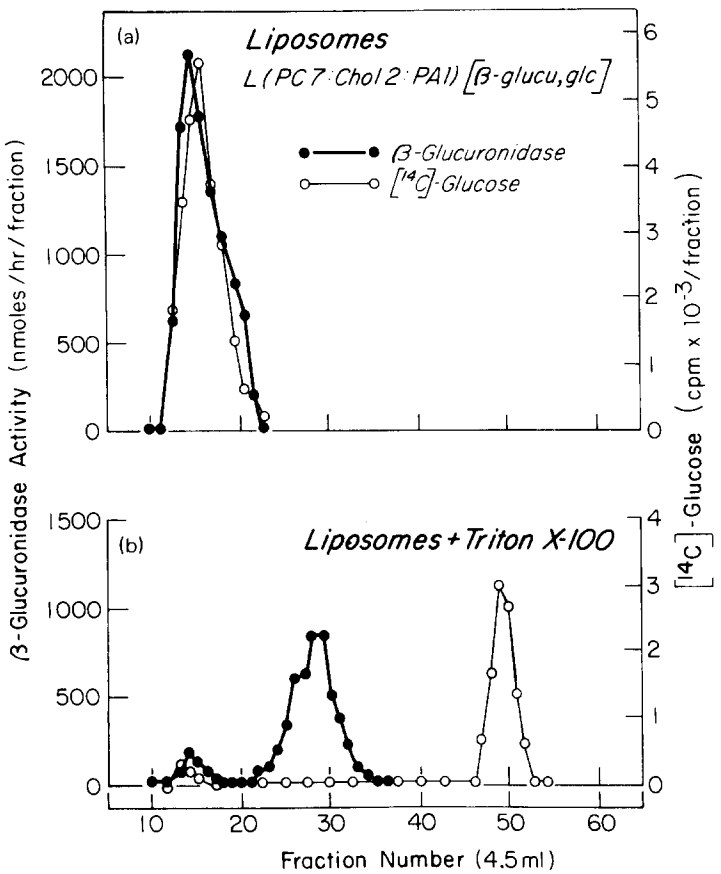


Fig. 2. Sepharose 6B chromatography of liposome-entrapped markers (a) before and (b) after treatment with Triton X-100. Bovine β-glucuronidase (●——●) and [$^{14}$C]glucose (○——○) were entrapped in negatively charged liposomes (phosphatidylcholine/cholesterol/phosphatidic acid, 7 : 2 : 1); the loaded liposomes were exhaustively washed to remove unentrapped markers, and then (a) either applied directly to the column or (b) disrupted with detergent and the chromatographed. For details, see text.

chromatographed on Sepharose 6B both β-glucuronidase and glucose were eluted in the void volume (Fig. 2a); in contrast, when the liposomes were treated with detergent and then chromatographed (Fig. 2b), the majority of the markers were eluted in positions corresponding to those observed for unentrapped β-glucuronidase and glucose, respectively.

Since the percentage of marker entrapped should increase as the lamellar surface charge is increased,[18] to create a greater interlamellar volume for entrapment, negatively charged liposomes were synthesized with various concentrations of phosphatidic acid. Table I shows that increasing the relative concentration of phosphatidic acid resulted in increased entrapment of β-glucuronidase and glucose in negatively charged liposomes. Similar results were obtained using positively charged liposomes in the above experiments. When negatively or positively charged liposomes were routinely prepared as described, about 5% of the total β-glucuronidase present in the buffer solution was entrapped; the remaining enzyme was recovered in the first centrifugation step and reused for other liposome preparations.

TABLE I

EFFECT OF INCREASED SURFACE CHARGE ON PERCENT ENTRAPMENT OF [$^{14}$C]GLUCOSE AND β-GLUCURONIDASE ACTIVITY

The percent entrapment is expressed as percent cpm of [$^{14}$C]glucose and nmol/h of β-glucuronidase activity entrapped of total available counts and activity, respectively; for details, see text.

| Liposome composition | Phosphatidic acid (mg) | Entrapped (%) | |
|---|---|---|---|
| Phosphatidylcholine: cholesterol: phosphatidic acid (molar ratio) | | [$^{14}$C]Glucose | β-Glucuronidase activity |
| 8 : 2 : 0 | 0 | 3.5 | 0.2 |
| 7 : 2 : 1 | 20 | 9.7 | 4.8 |
| 6.5 : 2 : 0.5 | 30 | 11.0 | 6.6 |

Further demonstration of marker entrapment was based on the fact that the anionic enzyme, β-glucuronidase, was efficiently entrapped in negatively charged liposomes whereas positively charged liposomes aggregated at pH 7.2, forming lipid-protein precipitates. Therefore, cationic liposomes were formed at pH 4.0, a pH unit below the pI of β-glucuronidase.

*Blood clearance and tissue distribution of bovine β-glucuronidase entrapped in negatively charged liposomes*

Fig. 3a illustrates the time course for the blood clearance and tissue uptake and distribution of bovine β-glucuronidase entrapped in negatively charged liposomes intravenously administered to β-glucuronidase-deficient mice. Approx. 90—100% of administered activity was recovered in murine tissues for up to 48 h post-injection. A rapid clearance of exogenous activity from the circulation ($t_{1/2} \approx 4$ min) was observed with a concomitant uptake of activity in several tissues, particularly the liver. The maximal hepatic recovery, approx. 75% of injected dose, was detected at 1 h, was retained for 48 h, and then gradually declined to non-detectable levels by 8 days. Approx. 20% of dose was recovered in the kidneys up to 4 days. Less than 5% of administered β-glucuronidase was found in splenic tissue at early time points and no activity was detected in the brain, heart, lungs or bone marrow.

The hepatic recoveries of intravenously administered bovine β-glucuronidase entrapped in negatively charged liposomes, buffer-loaded negatively charged liposomes and an in vitro mixture of unentrapped enzyme and buffer-loaded negatively charged liposomes are compared in Fig. 4. In contrast to the prolonged hepatic recovery of enzyme entrapped in negatively charged liposomes, the recovery of the mixture of enzyme plus buffer-loaded liposomes was identical to the time course of unentrapped enzyme alone. The identical time cources observed for unentrapped enzyme and the mixture of unentrapped enzyme and buffer-loaded liposomes further documents that enzyme was entrapped in negatively charged liposomes and was not administered as enzyme-lipid aggregates. No exogenous activity was detected in hepatic tissue following administration of buffer-loaded negatively charged liposomes.

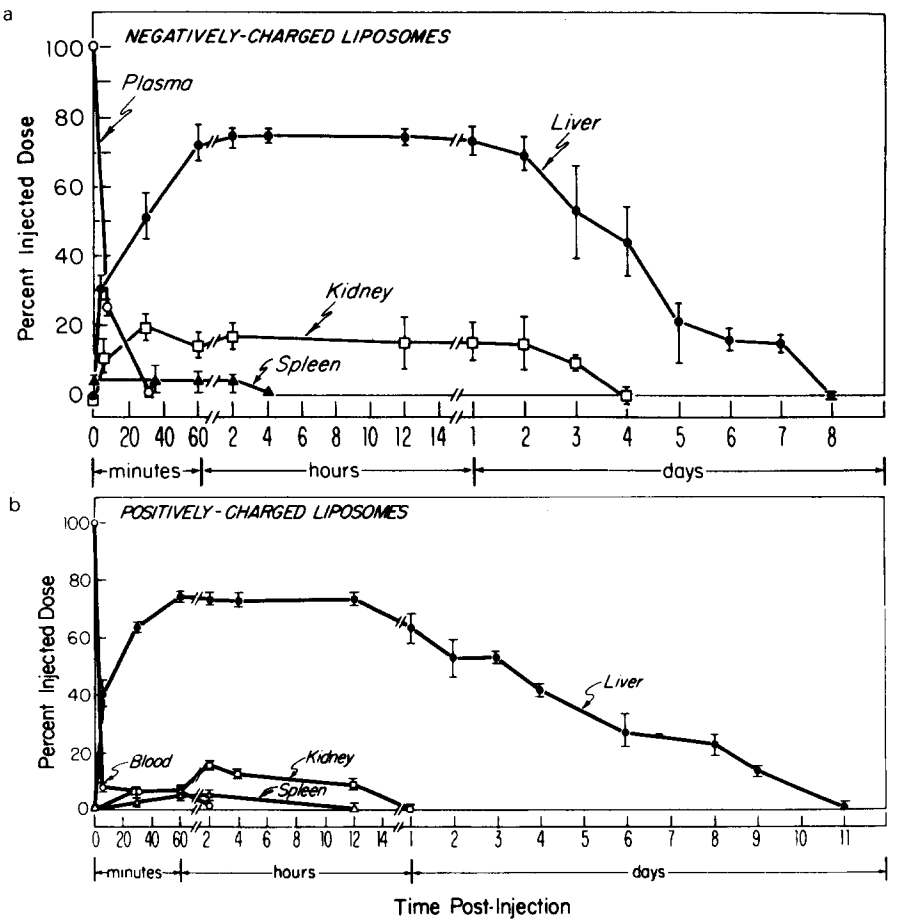


**Fig. 3. Comparison of the fates of bovine β-glucuronidase activity entrapped in (a) negatively charged liposomes (phosphatidylcholine/cholesterol/phosphatidic acid, 7 : 2 : 1) and (b) positively charged liposomes (phosphatidylcholine/cholesterol/stearylamine, 7 : 2 : 1) and administered intravenously to β-glucuronidase-deficient mice. Bovine β-glucuronidase activities recovered from murine blood and organs were determined as described in Materials and Methods and expressed as percentage of injected dose. Indicated values represent the mean and range of 4—10 mice at each time point.**

To determine the in vivo fate of the liposome carrier, [$^3$H]cholesterol was incorporated into negatively and positively charged liposomes and both enzymatic activity and radioactivity were monitored as shown for negatively charged liposomes in Fig. 5. The tritiated liposome membrane marker was detected in the liver by 5 min, was retained 8 h and then decreased linearly to low levels (5% of injected counts) by 3 days; in contrast, the exogenous activity was maintained in the liver at 70% of injected dose for 2 days before the levels decreased. A similar hepatic uptake was observed for the [$^3$H]cholesterol incorporated into enzyme-loaded positively charged liposomes; however, the radiolabel was retained at maximal levels for 24 h and then linearly decreased to low levels by 3 days. When only buffer was entrapped in labelled negatively charged liposomes, the radioactivity time course was markedly different than that observed for the enzyme-loaded liposomes. Radiolabelled buffer-loaded lipo-

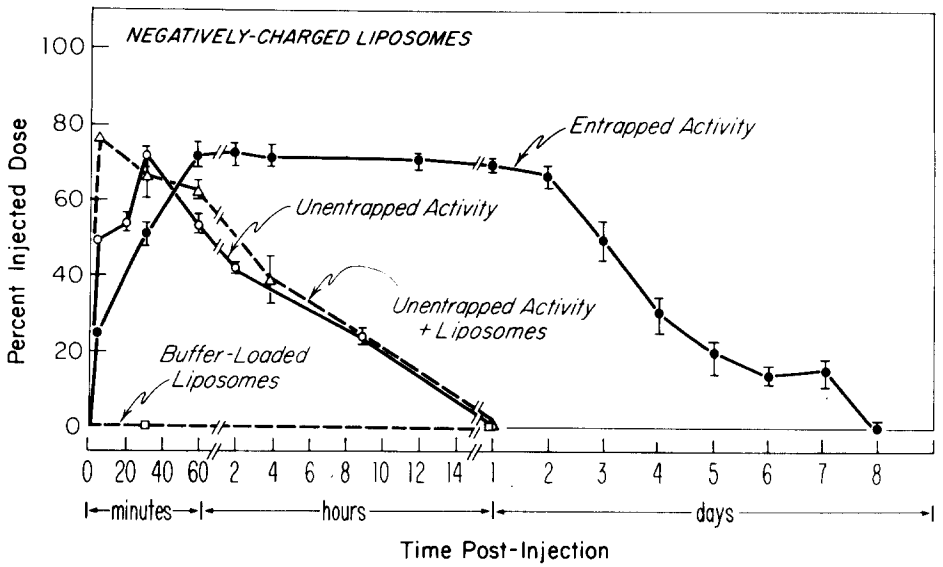


Fig. 4. Comparison of the hepatic recoveries of bovine β-glucuronidase activity entrapped in negatively charged liposomes (●———●), unentrapped bovine activity (○———○), and a mixture of bovine activity and buffer-loaded negatively charged liposomes (△- - - - -△). No thermolabile activity was detected after injection of only buffer-loaded negatively charged liposomes (□- - - - -□). Bovine β-glucuronidase activities recovered from hepatic tissues, as described in Materials and Methods, are expressed as percentage of injected dose (unentrapped dose = 1200 units; entrapped dose = 1000—1500 units). Negatively charged liposomes were composed of phosphatidylcholine/cholesterol/phosphatidic acid (7 : 2 : 1). Indicated values represent the mean and range of 3—8 mice at each time point.

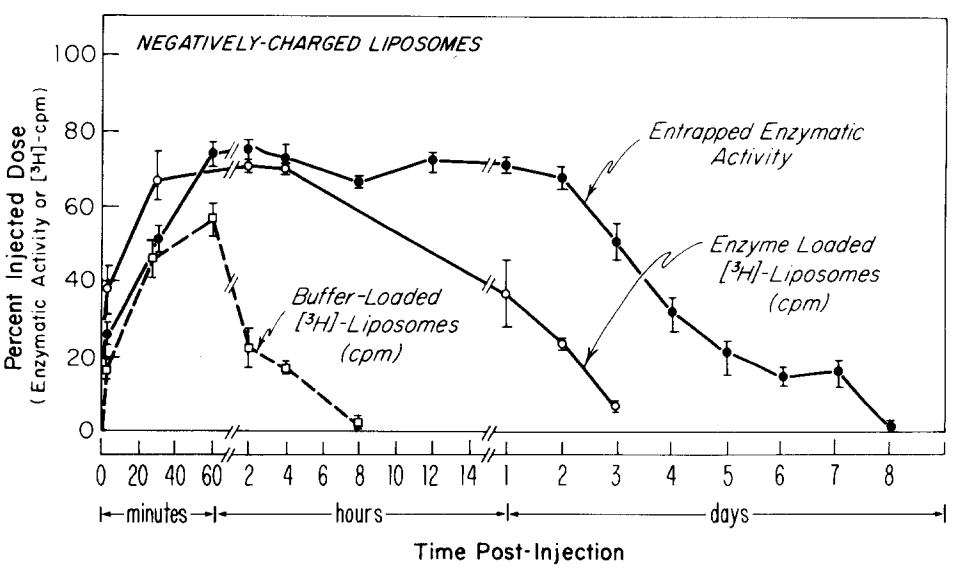


Fig. 5. Hepatic recoveries of bovine β-glucuronidase activity entrapped in [$^3$H]cholesterol-containing negatively charged liposomes (phosphatidylcholine/cholesterol/phosphatidic acid, 7 : 2 : 1). No thermolabile activity was detected after injection of only buffer-loaded [$^3$H]cholesterol-containing negatively charged liposomes. Bovine β-glucuronidase activity and radioactivity recovered from hepatic tissues, as described in Materials and Methods, are expressed as percentage of injected dose (negatively charged liposome entrapped enzymatic activity = 1000 units, [$^3$H]cholesterol dose = $5 \cdot 10^4$ cpm; buffer-loaded negatively charged liposomes [$^3$H]cholesterol dose = $5 \cdot 10^4$ cpm). Indicated values represent the mean and range of 3—8 mice at each time point.

TABLE II

HEPATIC SUBCELLULAR DISTRIBUTION OF RECOVERED BOVINE β-GLUCURONIDASE ACTIVITY ENTRAPPED IN NEGATIVELY CHARGED LIPOSOMES

Liver homogenates were fractionated as described in the text. The in vitro control mixture consisted of enzyme loaded in negatively charged liposomes and a liver homogenate.

| Source | Time post-injection (h) | | | | | In vitro control mixture |
|---|---|---|---|---|---|---|
| | 1 | 4 | 24 | 96 | 144 | |
| Total fractionated activity | (nmol/h) | | | | | |
| Murine | 3226 | 2418 | 3339 | 2250 | 3063 | 1903 |
| Bovine | 515 | 430 | 441 | 395 | 181 | 1051 |
| Subcellular fraction | (percent of total bovine activity) | | | | | |
| Nuclear | 5 | 7 | 8 | 6 | 20 | 2 |
| Mitochondrial-lysosomal | 76 | 70 | 75 | 69 | 66 | 10 |
| Soluble | 19 | 23 | 19 | 25 | 14 | 88 |

somes remained in the circulation longer than enzyme-loaded liposomes; 30% of injected [$^3$H]cholesterol was still present in the blood at 30 min post-injection compared to 6% of administered radiolabel in enzyme-loaded negatively or positively charged liposomes. Moreover, the [$^3$H]cholesterol marker was rapidly cleared from the liver within 8 h compared to the hepatic retention of the radiolabel incorporated into enzyme-loaded liposomes (Fig. 5).

*Hepatic subcellular distribution of bovine β-glucuronidase activity entrapped in negatively charged liposomes*

Table II shows the distribution of exogenous activity recovered in the nuclear, mitochondrial-lysosomal, and soluble fractions at various times post-injection. Total fractionated murine and bovine activities recovered in these experiments were all within 10% of the respective total activity in the whole homogenate. Approx. 70% of the total recovered bovine activity was detected in the lysosomally-enriched fraction at each time point studied. In contrast, 88% of the bovine activity was recovered in the soluble fraction when an in vitro control mixture of enzyme entrapped in negatively charged liposomes and a murine liver homogenate was fractionated, demonstrating the marked uptake of the administered activity in the lysosomally-enriched fraction.

*Blood clearance and tissue distribution of bovine β-glucuronidase activity entrapped in positively charged liposomes*

Fig. 3b shows the blood clearance and tissue uptake and distribution of bovine β-glucuronidase entrapped in positively charged liposomes. Exogenous activity was rapidly cleared from the circulation ($t_{1/2} \approx 2$ min) and concomitantly was taken up by the liver to a maximum level of 75% of dose by 1 h; the activity was retained at this level for 48 h and then gradually decreased to non-detectable levels by 11 days, similar to the fate of exogenous enzyme delivered in negatively charged liposomes. However, in contrast to the prolonged renal retention of activity entrapped in negatively charged liposomes, 10—15% of

TABLE III

HEPATIC SUBCELLULAR DISTRIBUTION OF RECOVERED BOVINE β-GLUCURONIDASE ACTIVITY ENTRAPPED IN POSITIVELY CHARGED LIPOSOMES

The in vitro control mixture consisted of enzyme loaded in positively charged liposomes and a murine liver homogenate. These values are representative of the results obtained at each time point ($n$ = 3—7). For details, see text.

| Source | Time post-injection (h) | | | | | | | | | In vitro control mixture |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 4 | 12 | 24 | 48 | 72 | 120 | 192 | |
| Total fractionated activity | (nmol/h) | | | | | | | | | |
| Murine | 4476 | 3962 | 4896 | 7530 | 4156 | 3762 | 5940 | 6050 | 4265 | 2898 |
| Bovine | 920 | 1944 | 1330 | 1017 | 1522 | 1173 | 1945 | 576 | 910 | 2652 |
| Subcellular fraction | (percent of total bovine activity) | | | | | | | | | |
| Nuclear | 12 | 6 | 12 | 2 | 1 | 7 | 8 | 17 | 13 | 28 |
| Mitochondrial-lysosomal | 47 | 48 | 32 | 37 | 33 | 23 | 22 | 57 | 60 | 10 |
| Soluble | 41 | 46 | 56 | 61 | 66 | 70 | 70 | 26 | 27 | 62 |

dose administered in positively charged liposomes was recovered in the kidneys for only 1 day; in addition, low levels of activity (approx. 5% of dose) were detected in the spleen and lungs up to 12 h post-injection. No exogenous activity was detected in brain, heart, or bone marrow.

*Hepatic subcellular distribution of bovine β-glucuronidase activity entrapped in positively charged liposomes*

Table III shows the recovery of exogenous activity in hepatic subcellular fractions following the administration of enzyme entrapped in positively charged liposomes. In contrast to the consistent and predominant lysosomal recovery of exogenous activity administered in negatively charged liposomes, a unique subcellular translocation of activity administered in positively charged liposomes was observed as a function of time post-injection. At early time points up to 4 h, the recovered exogenous activity was distributed about equally between the soluble and lysosomally-enriched fractions; however, from 12 to 72 h the activity was predominantly (70% of that recovered) localized in the soluble fraction. After 5 days the majority of recovered activity had shifted to the lysosomally enriched fraction.

The percent recovery of exogenous activity in the soluble fraction at 24 h increased proportionately with the amount of positively charged liposomes administered, presumably reflecting the effect of the increased amount of stearylamine administered. For example, when enzyme was administered in positively charged liposomes containing 0.285 and 0.475 mg of stearylamine, the soluble fractions contained 45 and 63% of recovered activity, respectively. Subcellular fractionation of an in vitro control mixture of enzyme entrapped in positively charged liposomes and a murine hepatic homogenate demonstrated

only 10% of the bovine activity in the lysosomal fraction, indicating that the in vivo patterns of subcellular distribution resulted from the unique interaction of enzyme-loaded positively charged liposomes with hepatic cells.

*Effects of negatively and positively charged liposomes on the hepatic subcellular distribution of endogenous lysosomal hydrolases*

The hepatic subcellular distributions of seven murine lysosomal hydrolases following the administration of exogenous β-glucuronidase entrapped in negatively and positively charged liposomes are compared in Table IV. These values

TABLE IV

DISTRIBUTION OF ENDOGENOUS LYSOSOMAL HYDROLASES IN THE MITOCHONDRIAL-LYSOSOMAL FRACTION FOLLOWING ADMINISTRATION OF ENZYME LOADED IN NEGATIVELY AND POSITIVELY CHARGED LIPOSOMES

Values are expressed as (mitochondrial-lysosomal/mitochondrial-lysosomal + soluble) X 100. Total recovered mitochondrial-lysosomal + soluble activities represent 80 ± 5% of the activities in the whole homogenate. Results are expressed in percent recovered in the mitochondrial-lysosomal fraction.

| Lysosomal hydrolase | Untreated control | Negatively charged liposomes | | | Positively charged liposomes | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Time post-injection (h) | | | Time post-injection (h) | | | | |
| | | 1 | 48 | 120 | 1 | 48 | 72 | 120 | 272 |
| α-Galactosidase | | | | | | | | | |
| Mean | 75 | 67 | 80 | 80 | 77 | 58 | 48 | 62 | 78 |
| Range | 71—81 | 67,67 | 79,81 | 77,83 | 72,84 | 54—64 | 45,52 | 61—63 | 75,80 |
| *n* | 7 | 2 | 2 | 2 | 2 | 3 | 2 | 3 | 2 |
| β-Galactosidase | | | | | | | | | |
| Mean | 71 | 69 | 68 | 67 | 72 | 55 | 50 | 65 | 74 |
| Range | 66—76 | 66—73 | 65,71 | 66,69 | 71,74 | 54—57 | 48—52 | 65—66 | 74,75 |
| *n* | 7 | 4 | 2 | 2 | 2 | 3 | 4 | 3 | 2 |
| β-Glucosidase | | | | | | | | | |
| Mean | 68 | 83 | 75 | 72 | 80 | 66 | 71 | 80 | 82 |
| Range | 63—75 | 81,86 | 74,76 | 72,73 | 78,83 | 60—74 | 70,72 | 79—82 | 81,84 |
| *n* | 7 | 2 | 2 | 2 | 2 | 3 | 2 | 3 | 2 |
| β-Glucuronidase | | | | | | | | | |
| Mean | 65 | 63 | 61 | 64 | 67 | 39 | 54 | 62 | 77 |
| Range | 62—72 | 61—65 | 57—65 | 64—65 | 65—69 | 38—44 | 51—59 | 60—65 | 76,78 |
| *n* | 7 | 10 | 10 | 10 | 7 | 5 | 6 | 3 | 2 |
| β-Hexosaminidase | | | | | | | | | |
| Mean | 82 | 79 | 79 | 80 | 77 | 61 | 61 | 68 | 80 |
| Range | 81—84 | 79—80 | 76,83 | 80,81 | 76,78 | 56—67 | 59—63 | 64—71 | 77,82 |
| *n* | 7 | 4 | 2 | 2 | 2 | 3 | 4 | 3 | 2 |
| α-Mannosidase | | | | | | | | | |
| Mean | 77 | 82 | 83 | 82 | 83 | 84 | 73 | 85 | 85 |
| Range | 72—81 | 82,82 | 81,86 | 80,84 | 82,84 | 82—87 | 70,76 | 84—87 | 84,87 |
| *n* | 7 | 2 | 2 | 2 | 2 | 3 | 2 | 3 | 2 |
| Arylsulfatase | | | | | | | | | |
| Mean | 60 | 61 | 50 | 64 | 56 | 56 | 64 | 65 | 68 |
| Range | 56—65 | 56,66 | 47,53 | 61,68 | 50,62 | 51—62 | 62,67 | 59—71 | 67,69 |
| *n* | 7 | 2 | 2 | 2 | 2 | 3 | 2 | 3 | 2 |

are expressed as the percent endogenous activity recovered in the mitochondrial-lysosomal fraction of the sum of recovered activity in the mitochondrial-lysosomal plus soluble fractions. Following administration of negatively charged liposomes, the subcellular distribution of all seven endogenous activities were essentially the same as those for the untreated murine control homogenates. However, the administration of enzyme entrapped in positively charged liposomes resulted in a significant change in the distribution of these lysosomal markers. Although the distribution of endogenous lysosomal hydrolases was similar to those of untreated mice at 1 h, a marked shift from the predominant lysosomal to a soluble localization was particularly evident at 1—3 days post-injection. For example, the differences in the percent of endogenous activity recovered in the lysosomally-enriched fraction at 1 and 48 h for β-glucuronidase, α-galactosidase, β-galactosidase and β-hexosaminidase were 28, 19, 17 and 16%, respectively, indicating a translocation of these lysosomal hydrolases into the cell cytoplasm.

Stearylamine did not inhibit any endogenous hydrolase activities when in vitro mixtures of a fractionated control liver and varying concentrations of stearylamine (0.01, 0.1 and 1 mg) were incubated for 30 min at 37° C prior to assay. Moreover, the possibility that stearylamine was inhibiting hydrolase activity in vivo was eliminated by checking the specific and total recovered activities for the hepatic subcellular fractions of all enzymes assayed following the administration of enzyme entrapped in negatively charged liposomes, which were all within the control range of untreated mice.

## Discussion

The major purpose of this investigation was to compare the in vivo tissue and subcellular fates of enzyme delivered in negatively and positively charged liposomes as well as to determine the effects of these enzyme-loaded lipid vesicles on cellular physiology, specifically lysosomal integrity. Enzyme and/or marker entrapment in both negatively and positively charged liposomes was demonstrated (Figs. 1, 2a and 2b; Table I) according to the criteria of Weissmann et al. [18]. The percent of β-glucuronidase entrapped in the routine liposome preparations was comparable to values previously reported for other macromolecules in negatively [18] or positively charged liposomes [31].

The blood clearances and tissue distributions of bovine β-glucuronidase administered in negatively charged liposomes to β-glucuronidase-deficient mice were similar to those previously reported by Gregoriadis and Ryman [19] for yeast β-fructofuranosidase administered to rats in negatively charged liposomes. In both studies the majority of exogenous enzyme was detected in hepatic lysosomes. However, when negatively and positively charged liposomes were loaded with β-glucuronidase, administered to the deficient mice, and their in vivo fates compared, two important differences were noted. First, in contrast to the minimally detectable recovery of exogenous activity administered in positively charged liposomes or administered unentrapped [10], enzyme entrapped in negatively charged liposomes was recovered in renal tissue at about 15—20% of dose for up to 4 days. These findings suggest that negatively charged liposomes may be useful for the delivery of macromolecular therapeutic agents for dis-

orders with renal involvement and indicate the importance of the liposome composition and surface charge for targeting entrapped molecules to specific tissue sites. Second, the intracellular retention of exogenous activity in liver, the major site of tissue uptake, was different. In contrast to the rapid hepatic clearance of unentrapped activity (undetectable by 1 day), enzyme delivered in negatively charged liposomes was detected up to 8 days and even more remarkably, activity entrapped in positively charged liposomes was detected for up to 11 days. The prolonged hepatic recovery of exogenous enzyme delivered in positively charged liposomes may reflect the inhibitory effect of stearylamine on phospholipases which degrade the lecithin in the liposomal membrane. In fact, phosphatidic acid (used in negatively charged liposomes) has been shown to stimulate bacterial phospholipase B and plant phospholipase D activities [32], while stearylamine and other cationic amphipaths are strong inhibitors of phospholipase activity [33]. Thus, the stearylamine in positively charged liposomes may serve to retard the release and metabolic availability of entrapped enzyme by phospholipase inhibition, thereby extending the intracellular retention of administered enzymatic activity.

To investigate the in vivo fate of liposome-entrapped enzyme, and in particular the kinetics of liposome disruption and enzyme release, radiolabelled cholesterol was incorporated into the liposomal membrane and the hepatic uptake and clearance of radioactivity and exogenous activity were simultaneously determined. The concomitant hepatic uptake of enzyme and the liposomal marker evidence uptake of intact enzyme-loaded liposomes. Exogenous activity was maintained at maximal hepatic levels as the radiolabel was slowly cleared over 3 days. These data suggest that the negatively charged liposomes were taken up intact by hepatic lysosomes and that the multilamellar liposomes were slowly disrupted, releasing the radiolabel for exchange and clearance and the enzyme for metabolic availability. Latency of the liposome-entrapped activity was not detectable since our enzyme assay does not distinguish between entrapped and released activity.

The more rapid uptake and prolonged retention of radiolabel administered in enzyme-loaded liposomes compared to those of buffer-loaded liposomes (Fig. 5) indicates that enzyme entrapped in negatively charged liposomes was taken up and processed by the hepatic lysosomal apparatus differently. These findings suggest that some of the entrapped proteins were partially exposed on the liposome surface. The exposed molecules may account for the observed increased hepatic uptake, similar to that found for unentrapped enzyme. In addition, and analogous to the glycoprotein recognition signals reviewed by Ashwell and Morrell [34], the exposed glycoprotein enzyme may signal a specific hepatic recognition and processing mechanism which might account for the slower clearance of the enzyme-loaded liposomal membrane label.

Subcellular fractionation of hepatic tissue further characterized the in vivo fate of enzyme entrapped in negatively or positively charged liposomes. The activity entrapped in negatively charged liposomes was primarily localized in the lysosomal fraction throughout the time course, similar to that observed for unentrapped enzyme; however, more than half of the activity entrapped in positively charged liposomes was recovered in the soluble fraction during the first few days following injection. These results suggest that entrapment of enzymes

in negatively charged liposomes may provide an appropriate vehicle for enzyme replacement endeavors in selected lysosomal storage diseases, whereas positively charged liposomes might be useful for the delivery of enzymes or other therapeutic agents to the cytoplasm. More importantly, and surprisingly, the hepatic subcellular distribution of enzyme administered in positively charged liposomes changed during the 11 day time course. At the earliest time point studied, the lysosomally enriched fraction contained most of the exogenous β-glucuronidase activity suggesting that positively charged liposomes were initially taken up by the lysosomal apparatus; during the following 3 days, the majority of recovered exogenous activity was translocated to the soluble fraction. The exogenous activity initially detected in the soluble fraction (36% of recovered activity) may have resulted from a proportion of enzyme-loaded positively charged liposomes which fused with the cell membrane and released their contents into the cytoplasm. This possibility is consistent with the recent report by Poste and Papahadjopoulos [35] who demonstrated that the mechanisms of liposome uptake by cultured fibroblasts (endocytosis versus membrane fusion) was dependent on the liposomal lipid composition. Alternatively, this soluble activity may have been released from positively charged liposomes which already had been disrupted by contact with the lysosomal apparatus. Therefore, it was reasoned that the simultaneous characterization of the subcellular distribution of the exogenous activity and several endogenous murine lysosomal hydrolase activities might provide insight into the mechanism responsible for the observed subcellular translocation of enzyme administered in positively charged liposomes.

Indeed, the subcellular distribution of four murine lysosomal hydrolases was also observed to shift to the soluble fraction with a time course similar to that of enzyme delivered in positively charged liposomes. In contrast, administration of enzyme entrapped in negatively charged liposomes did not alter the lysosomal localization of any of the endogenous hydrolases assayed. These findings suggest that stearylamine, the positively charged lipid, may have altered the lysosomal membrane and caused the release of endogenous hydrolases. The fact that there was a proportional increase of the endogenous lysosomal activities recovered in the soluble fraction when the amount of stearylamine administered was increased was in support of this implication. The failure to find all of the endogenous activities increased in the soluble fraction may be in part a function of their association with the lysosomal membrane; in fact, both β-glucosidase [36] and α-mannosidase [37] are known to have membrane-bound activities. Another explanation might be that these hydrolases occur predominantly in parenchymal cell types which reportedly [16] are not involved in liposome uptake. Thus, it can be deduced from these studies that initially the positively charged liposomes were in part localized in lysosomes, and that the subsequent disruption of the liposomal lamellae allowed the positively charged component, stearylamine, to alter lysosomal integrity and cause the release of exogenous and endogenous hydrolases into the cytoplasm. The recent report of toxic effects of bulging and deformation of the cell membrane observed after treatment of cultured HeLa cells with various doses of stearylamine-containing liposomes [17] provides additional support for the dose-dependent toxicity of stearylamine on cell membranes. It is not unlikely that

other positively charged lipids in liposomes may cause similar alterations of lysosomal integrity. Although the injected mice did not show any clinically detectable manifestations, the potential deleterious effects of hydrolase release on the cytoplasmic milieu remains unknown and requires further investigation.

These studies emphasize the importance of evaluating the physiological effects of administered macromolecules in a mammalian model system prior to human trials. Liposomes have been suggested as carriers for the intracellular delivery of therapeutic macromolecules [9,16—21]; already preliminary human trials have focused attention on the potential of these biodegradable vesicles to target and protect therapeutic agents [5,38]. Our data support the use of liposomes as effective means of both prolonging tissue retention and delivering enzyme to various tissue sites. However, the possible physiological consequences of liposome administration have not been previously evaluated. Our studies demonstrate that the physiologic lipids used to entrap bovine β-glucuronidase in negatively charged liposomes did not alter lysosomal function based on their effect on the subcellular distribution of endogenous lysosomal markers. In contrast, the stearylamine in positively charged liposomes presumably caused a temporary labilization of the lysosomal membrane. In addition, our data indicating the presence of exposed protein on the liposomal surface suggest that the immunosurveillance system might recognize the exogenous enzyme as foreign thus eliminating an "immunoprotective" attribute of entrapment. Furthermore, other reports identify the possible immunological hazards of entrapped proteins and glycoproteins partially exposed on liposomal surfaces [39,40]. Thus, these studies are instructive and indicate the necessity to evaluate the potential physiological and immunological complications which may result from the administration of specific compositions of liposomes before clinical trials are undertaken.

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