ENZYME ENTRAPMENT IN LIPOSOMES

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1. Introduction

Enzyme replacement therapy for patients with various disorders in which a specific enzyme activity is absent from one or more tissues, has been attempted in several instances by direct administration to the patient of an enzyme designed to remove undesirable accumulation products [1–5]. However, apart from the possible immunological response that could arise from the foreign protein, there are also the problems related to undesirability of having certain enzymes in the circulation and of directing the given protein to a particular tissue.

We thought that some of the difficulties mentioned could be circumvented by entrapping proteins into liposomes (lipid spherules). Liposomes are formed when phospholipids are allowed to swell in aqueous media and become hydrated liquid crystals. These, when suitably dispersed, consist of a series of concentric bilayers which alternate with aqueous compartments in which can be entrapped water-soluble substances [6, 7].

The present paper describes the entrapment of *Aspergillus niger* amyloglucosidase (E.C.3.2.1.3.) and albumin into liposomes. The choice of the amyloglucosidase in this entrapment investigation reflects our interest in the glycogen storage diseases, while the commercial availability of ^{131}I-albumin has provided a readily detectable protein, similar in both molecular size and charge to the amyloglucosidase.

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2. Materials and methods

Egg phosphatidylcholine (lecithin) was a gift from Dr. W. Tampion and Mr.Q.F. Akhong of this Department. It was supplied in ampoules (40 μmoles in chloroform) individually sealed under N_2. The phospholipid was chromatographically homogeneous and was not peroxidized [8]. Cholesterol was obtained from BDH Chemicals; dicetyl phosphate from K. and K. Laboratories; Triton X-100 from Sigma Chemical Company; mannitol from Fisons Scientific Apparatus Ltd., Sepharose 6B from Pharmacia, and soluble starch from Hopkin and Williams Ltd. *Aspergillus niger* amyloglucosidase (12 IU/mg [9]) and human serum albumin (Kekwick and Mackay, M.R.C., spec. Ref. ser. N286. 1954 from the Lister Institute, London) were gifts from Professor W.J. Whelan, University of Miami, USA. Human serum ^{131}I-albumin (30 μCi/mg) was purchased from Radiochemical Centre, Amersham. All other reagents were of analytical grade.

Preparation of liposomes. Lecithin (40.0 μmoles), cholesterol (11.4 μmoles) and dicetyl phosphate (5.7 μmoles) giving a molar ratio of 7:2:1 [10] were dissolved in 5 ml chloroform in a round-bottomed flask. Rotary evaporation at 37° resulted in the formation of a thin film which was immediately dispersed by gentle shaking under N_2 in 4 ml of 3.3 mM phosphate buffer, pH 7.2, containing varying amounts of amyloglucosidase or albumin (mixed with a trace of iodinated albumin). The milky suspension was kept at room temperature for about 2 hr and then soni-

cated under a stream of N_2 for 10 sec at 4° using a 1.8 cm titanium probe at 1.5 A in an MSE 60 W sonicator. The suspension (which becomes almost clear on sonication) was then kept at room temperature for another 2 hr and passed through a Sepharose 6B column (36 X 2.7 cm) equilibrated with 6.7 mM phosphate buffer, pH 7.2, containing 0.9% NaCl. Liposomes were eluted at the end of the void volume in about 15 ml and were usually centrifuged in an MSE Superspeed 50 centrifuge at 100,000 *g* for 60 min. The pellet was suspended in 1–2 ml buffered saline and kept at 4°. Alternatively, following sonication, the liposomal preparation was centrifuged at 100,000 *g* for 2.5 hr. The supernatant containing the non-entrapped protein was re-used in the preparation of a new batch of protein-containing liposomes, and the pellet was suspended in 4 ml buffered saline and passed through the Sepharose 6B column. The liposomes were again collected at the end of the void volume.

In some experiments, designed to test for true entrapment of protein rather than non-specific association of protein with liposomes, liposomes were prepared in the absence of protein. Amyloglucosidase or ^{131}I-albumin dissolved in 0.5 ml 3.3 mM phosphate buffer, pH 7.2 was then mixed with the liposome suspension 1–2 hr before passage through the column or centrifugation.

Lipid in the fractions collected from the column was determined by measuring the apparent absorbance at 410 nm [10]. Amyloglucosidase activity in 0.1 ml liposome preparation was measured using starch as substrate [9]. The liberated glucose was determined enzymatically using glucose oxidase [11, 12]. Latent amyloglucosidase activity was measured following incubation of samples at 37° for 30 min with an equal volume of a 1% solution of Triton X-100. Radioactivity of ^{131}I-albumin was assayed in a Wallac γ-counter (GTL 300-1,000).

3. Results

Formation of liposomes in the presence of amyloglucosidase or albumin, results in the partial entrapment of the proteins. Separation of the entrapped protein from the excess free protein occurred on passage of the liposomal preparation through a Sepharose 6B column (figs. 1 and 2). Most of the amyloglucosidase

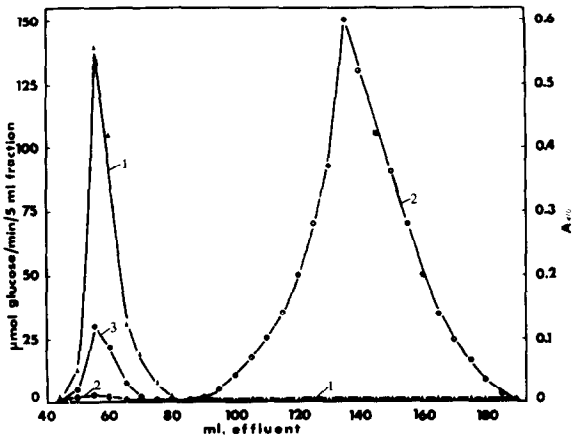

Fig. 1. Separation of liposomes containing entrapped amyloglucosidase from free enzyme on Sepharose 6B. Curve 1: lipid (A_{410}); curve 2: free enzyme; curve 3: latent enzyme, i.e. after Triton X-100 treatment (see text).

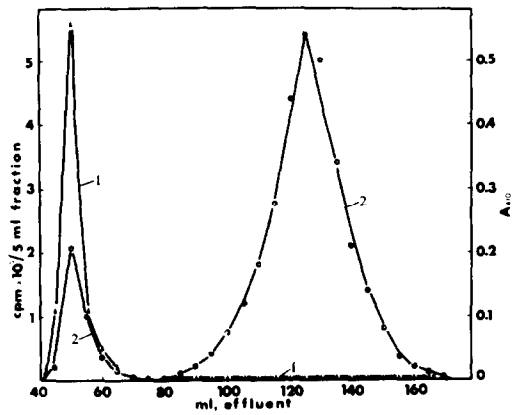

Fig. 2. Separation of liposomes containing ^{131}I-albumin from free ^{131}I-albumin on Sepharose 6B. Curve 1: lipid (A_{410}); curve 2: cpm ^{131}I-albumin.

activity associated with liposomes (fig. 1) is latent and can be measured only after destruction of the liposomal membrane structure with Triton X-100. This detergent is non-inhibitory to the enzyme activity determination at the concentration used. Mixing of the enzyme with liposomes prepared in the absence of the enzyme, 1–2 hr before column chromatography, does not result in any appreciable activity in the liposomal fraction. Thus, amyloglucosidase appears to be captured in liposomes rather than being

Table 1
Entrapment of amyloglucosidase and ^{131}I-albumin during the preparation of liposomes.

Protein (mg)	% Entrapment
Amyloglucosidase	
4	4.0
30	3.6
60	4.0
60	5.5
60	6.5
^{131}I-Albumin	
2	6.8
30	6.9
60	7.0
60	7.2
60	10.6

Table 2
Recovery of liposomes after centrifugation (100,000 *g* for 60 min) of eluate from Sepharose 6B column.

Protein*(mg)	Recovery in pellet after centrifugation (% of total in liposomes)	
	Protein	Lipid
Amyloglucosidase		
30	91.3	95.1
60	95.6	97.2
^{131}I-Albumin		
30	92.6	96.3
60	97.2	98.1

* Amount used in preparation of liposomes.

associated with them through bonding. The procedures used in the preparation of liposomes did not result in loss of activity of the enzyme and the liposomes could be stored at 4° in the buffered saline without loss of enzyme activity.

Entrapment was also observed with ^{131}I-albumin (fig. 2) and, as in the case of amyloglucosidase, mixing of ^{131}I-albumin with liposomes prepared in the absence of ^{131}I-albumin, prior to column chromatography, failed to incorporate any appreciable radioactivity in the eluted liposomal fractions. The conditions used in the entrapment of albumin do not favour binding of the protein to liposomes [13].

The efficiency of protein entrapment under the present conditions is shown in table 1. Preparation of liposomes in the presence of varying amounts of amyloglucosidase resulted in a 4–6.5% entrapment of the enzyme. This corresponds to about 2.4–3.6 mg of enzyme entrapped within liposomes. In the case of albumin the entrapment efficiency is higher.

Centrifugation of the protein-containing liposomal eluate at 100,000 *g* for 60 min resulted in the sedimentation of the liposomes (table 2). More than 90% of the amyloglucosidase activity or albumin radioactivity, and more than 95% of the lipid, were recovered in the pellet which could then be resuspended in a minimal volume of buffered saline.

4. Discussion

Incorporation of proteins into liposomes has already been reported [14, 10]. Sessa and Weissman [10] in their attempt to construct a model of organelles, namely lysosomes, were able to incorporate lysozyme into liposomes. Our efforts, however, are directed towards the use of liposomes as therapeutic agents, namely enzyme carriers in enzyme replacement therapy.

In this paper, the possibility of entrapping amyloglucosidase, a fungal enzyme which splits the $1 \rightarrow 4$ and $1 \rightarrow 6$ bonds of glycogen [15, 16], and iodinated albumin, was examined.

Preparation of liposomes in the presence of amyloglucosidase or albumin resulted in partial entrapment of the protein. Separation of the protein-containing liposomes from the excess free protein was achieved by using a Sepharose 6B column (figs. 1 and 2). Most of the amyloglucosidase activity associated with liposomes was latent in that it could only be detected after treatment with Triton X-100 which is known to disrupt the closed lamellar configuration of the liposomes. Such a configuration is shown in fig. 3 which is an electron micrograph of negatively-stained liposomes prepared in the presence of amyloglucosidase. The enzyme is presumably segregated within the aqueous compartments between the lipid lamellae [10].

Centrifugation of the liposomal eluates at 100,000 *g* does not affect the integrity of liposomes (table 2) and ensures the availability of the preparation in a small volume.

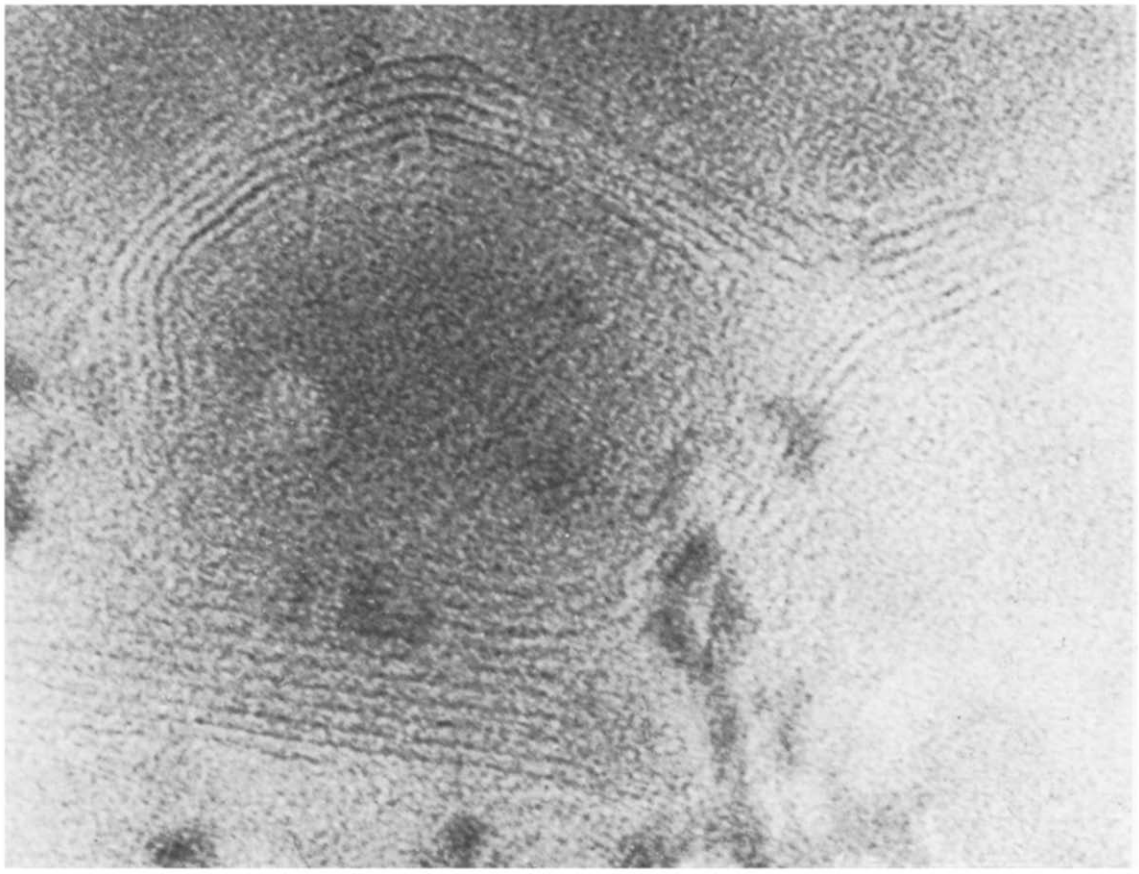

Fig. 3. Electron micrograph of a negatively stained liposome in which concentric lipid bilayers separated by electron-opaque layers can be seen. Negatively stained with 2% sodium phosphotungstate on carbon film, × 432,000 (Bar = 50 nm). The micrograph was kindly prepared by Dr. J. Wrigglesworth, Department of Biochemistry, Chelsea College of Science and Technology, London.

The extent of entrapment of a protein in liposomes is for obvious reasons directly related to the efficiency of these particles as enzyme replacement therapy agents. We therefore prepared liposomes under conditions which would favour extensive entrapment. The factors which affect the volume of the aqueous compartments in between the lipid lamellae and in which the captured protein is segregated [6] are influenced by the molar percentage of the charged lipids, and the ionic strength of the environment [6, 17]. Examination of the amyloglucosidase by cellusose acetate electrophoresis showed that it carried a negative charge at pH 7.2. The inclusion of dicetyl phosphate in the liposome structure minimizes the adsorption of the enzyme on to the liposomal surface [10] and increasing amounts of dicetyl phosphate were found proportionally to improve the entrapment yield. However, no improvement occurred at concentrations of dicetyl phosphate above that used in the present experiments (10% of the total molarity of the lipids used). Similar findings have been reported for sterylamine [10]. Decreasing ionic strength was also found to improve entrapment yield. It was observed, however, that replacement of the phosphate buffer with 300 mM mannitol, which gave an ionic strength much lower than that used in the present experiments, brought about the precipitation of amyloglucosidase upon sonication of the liposomal preparation. Finally, decrease of sonication time down to 10 seconds contributed to a more extensive entrapment of the proteins.

The entrapment of amyloglucosidase and iodinated albumin into liposomes offers the possibility for the use of phospholipid spherules (not necessarily of the precise composition employed in this investigation) as enzyme carriers in enzyme replacement therapy. The fate of the protein-containing liposomes upon their administration to animals is currently under investigation.

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