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Lysosomal Localization of β-Fructofuranosidase-Containing Liposomes Injected into Rats

SOME IMPLICATIONS IN THE TREATMENT OF GENETIC DISORDERS

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Yeast β-fructofuranosidase (invertase) or ^{131}I-labelled albumin were entrapped into liposomes composed of phosphatidylcholine, cholesterol and phosphatidic acid. Of the β-fructofuranosidase activity in the liposomal preparations 96–100% was latent. The following observations were made in experiments with rats injected with protein-containing liposomes. 1. After injection of β-fructofuranosidase-containing liposomes (220 units or 1.5mg of β-fructofuranosidase and 17.5mg of lipid), β-fructofuranosidase activity in blood retained its latency but the activity declined to 50% of the injected dose in 1h. Within 6h much of this activity was recovered in the liver and spleen (respectively 45% and 10% of that injected). For up to 21h after injection, the mitochondrial–lysosomal fraction was the principal location of the hepatic β-fructofuranosidase activity. 2. Lysosomal localization of liposomal protein was supported by the observed increase in the trichloroacetic acid-soluble radioactivity during incubation of the lysosome-rich fraction of the liver of rats injected with liposomes containing ^{131}I-labelled albumin. 3. Association of liposomal protein with lysosomes was demonstrated on subfractionation of the mitochondrial–lysosomal fraction of the liver of rats injected with β-fructofuranosidase-containing liposomes in a Ficoll–mannitol gradient. β-Fructofuranosidase, lysosomal and mitochondrial enzyme marker activities were found to exhibit similar distribution patterns along the gradient. However, in similar experiments with rats previously injected with Triton WR-1339 or dextran (known to alter the specific gravity of lysosomes), only β-fructofuranosidase and lysosomal marker moved along the gradient, in strikingly similar patterns. 4. The lysosomal localization of injected liposome-entrapped material can probably be utilized in the treatment of certain disorders in man.

We have recently reported the entrapment of *Aspergillus niger* amyloglucosidase (EC 3.2.1.3) and albumin into liposomes made of egg phosphatidylcholine, cholesterol and dicetyl phosphate (Gregoriadis *et al.*, 1971). Liposomes consist of a series of concentric bilayers of lipids alternating with aqueous compartments within which soluble substances can be entrapped (Bangham, 1968; Sessa & Weissman, 1968). Studies *in vivo* in rats with radioactively labelled amyloglucosidase or albumin-containing liposomes (Gregoriadis & Ryman, 1971, 1972) showed that the proteins and their carrier are rapidly taken up by the liver and spleen. By subcellular fractionation of the liver it was found that the mitochondrial–lysosomal fraction is the principal site of liposome location within the cell. Further, the immunochemically observed hepatic catabolism of amyloglucosidase suggested that lysosomes might participate in the catabolism of the enzyme, presumably after rupture of the liposomal membranes (Gregoriadis & Ryman, 1972).

In the present experiments, β-fructofuranosidase (EC 3.2.1.26) or ^{131}I-labelled albumin were entrapped into liposomes [made of phosphatidylcholine, cholesterol and phosphatidic acid, the last replacing the unphysiological dicetyl phosphate employed in previous preparations (Gregoriadis *et al.*, 1971; Gregoriadis & Ryman, 1972)] and injected into rats. The high specific activity of β-fructofuranosidase has enabled us, for the first time, to follow the fate of enzyme activity rather than enzyme radioactive label or immunological identity (Gregoriadis & Ryman, 1972). Gradient fractionation of the mitochondrial–lysosomal fraction of the liver of rats injected with β-fructofuranosidase-containing liposomes, together with incubation studies of the

lysosome-rich fractions of the liver of rats injected with liposomes containing ^{131}I-labelled albumin, revealed that the two proteins are associated with the lysosomes.

Materials and Methods

Chemicals

Chromatographically homogeneous phosphatidic acid was a gift from Dr. W. Tampion and Mr. Q. F. Ahkong of this Department. It was prepared by the method of Papahadjopoulos & Miller (1967) and supplied in ampoules (30.0 μmol in $CHCl_3$) individually sealed under N_2. Egg phosphatidylcholine (grade I) was purchased from Lipid Products, Epsom, Surrey, U.K., and sucrose from BDH Chemicals Ltd., Poole, Dorset, U.K.; Triton WR-1339 was from Ruger Chemical Co., Irvington-on-Hudson, N.Y., U.S.A.; baker's-yeast β-fructofuranosidase (grade VI), dextran type 200C (average mol.wt. 204000) and D-mannitol were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; Triton X-100 was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; Ficoll (mol.wt. by light-scattering, 400000) and Sepharose 6B were from Pharmacia, Uppsala, Sweden. Human serum albumin, ^{131}I-labelled human serum albumin, dicetyl phosphate, cholesterol, [1α,2α-^{3}H]cholesterol and all chemicals used for the measurement of *N*-acetyl-β-D-glucosaminidase activity and succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase activity have been described elsewhere (Gregoriadis & Ryman, 1972).

Preparation of protein-containing liposomes

The procedure used for the preparation of liposomes containing β-fructofuranosidase or ^{131}I-labelled albumin, although on a larger scale, was essentially the same as that applied for amyloglucosidase-containing liposomes (Gregoriadis *et al.*, 1971). However, the unphysiological dicetyl phosphate employed in earlier liposomal preparations (Gregoriadis *et al.*, 1971; Gregoriadis & Ryman, 1972) was replaced by phosphatidic acid. In short, 200 μmol of egg phosphatidylcholine, 57 μmol of cholesterol and 28.5 μmol of phosphatidic acid (molar proportions 7:2:1) were dissolved in 25ml of chloroform in a 1-litre round-bottom flask. The thin film formed on the walls of the flask after rotary evaporation at 37°C was dispersed by gentle shaking under N_2 in 20ml of 3.3mM-potassium phosphate buffer, pH7.2, containing 500mg of β-fructofuranosidase or 500mg of albumin mixed with 2–3mg of ^{131}I-labelled albumin. Then 2h later the milky suspension was sonicated for 45s at 4°C by the use of a 1.8cm titanium probe at 1.5A in an MSE 60W sonicator and kept at room temperature for 2h. The protein-containing liposomes were separated from non-entrapped protein by passage through a Sepharose 6B column (65cm×4cm) equilibrated with 6.7mM-potassium phosphate buffer, pH7.2, containing 0.9% NaCl (buffered saline). Protein-containing liposomes were eluted at the end of the void volume in about 50ml and were centrifuged at 100000*g* for 60min. The pellet was suspended in 5ml of buffered saline, assayed for lipid content (Gregoriadis *et al.*, 1971) and kept under N_2 at 4°C. [1α,2α-^{3}H]Cholesterol-labelled liposomes containing ^{131}I-labelled albumin (lipid composition: phosphatidylcholine, [1α,2α-^{3}H]-cholesterol and dicetyl phosphate) were prepared as described previously (Gregoriadis & Ryman, 1972). For experiments *in vivo*, liposomes were used 2–7 days after their preparation.

Animal experiments

Male albino rats (Wistar) weighing approx. 100g were used throughout. They were injected in the tail vein with 0.5ml of β-fructofuranosidase-containing liposomes, or, for control animals, with 0.5ml of buffered saline, and decapitated at various time-intervals. In some experiments, 1.0ml of 0.9% NaCl alone or 0.9% NaCl containing 200mg of Triton WR-1339 or 200mg of dextran was given to rats intraperitoneally 3.5 days before the injection of β-fructofuranosidase-containing liposomes. In one experiment, five rats were injected with 0.5ml of liposomes containing ^{131}I-labelled albumin and killed after 30min, and in another, two rats were injected with 1ml of [1α,2α-^{3}H]cholesterol-labelled liposomes containing ^{131}I-labelled albumin and killed after 30min. At the end of the experiments, the livers (perfused through the hepatic portal vein with 0.9% NaCl until they became a pale olive colour) and the spleens were excised, washed with 0.9% NaCl, blotted in filter paper and weighed. For the measurement of β-fructofuranosidase activity, part of the liver and the whole spleen were homogenized in cold water (5–10ml of water/g of tissue).

Fractionation of the liver of rats injected with β-fructofuranosidase-containing liposomes

A portion (2–5g) of the liver of the animals injected with β-fructofuranosidase-containing liposomes was homogenized in 0.3M-mannitol (5ml/g of liver) and fractionated to nuclear (N), mitochondrial–lysosomal (M+L), microsomal (P) and soluble (S) fractions (Gregoriadis & Sourkes, 1967). In other experiments the liver of untreated rats was homogenized in 0.3M-mannitol in which 0.1ml of β-fructofuranosidase-containing liposomes was previously suspended. Fractionation of the latter homogenate was carried out 60min later. All particulate fractions obtained were resuspended in 0.3M-mannitol up to a volume of 5–10ml.

Incubation of the mitochondrial–lysosomal (M+L) fraction from the liver of rats injected with liposomes containing ^{131}I-labelled albumin

Livers from animals injected with liposomes containing ^{131}I-labelled albumin were individually homogenized in 0.3M-sucrose (5ml/g of liver) and then fractionated by differential centrifugation to obtain the M+L fraction (Gregoriadis & Sourkes, 1967). This fraction was carefully resuspended in 0.01M-tris–HCl buffer, pH7.4, containing 0.3M-sucrose (buffered sucrose) (5ml/g of liver). A small volume of this suspension was kept for assay of radioactivity and the remainder divided into two equal parts. To one part 1.0ml of Triton X-100 solution in buffered sucrose was added (final concn. 0.1%) and to the other 1.0ml of buffered sucrose. Both suspensions were immediately incubated at 22°C (Davies *et al.*, 1969). In another experiment, the M+L fraction from the liver of an untreated rat was resuspended in buffered sucrose (5ml/g of liver) previously mixed with 0.1ml of liposomes containing ^{131}I-labelled albumin. At various time-intervals, samples were removed from all suspensions, mixed with an equal volume of 20% (w/v) trichloroacetic acid and centrifuged in a bench centrifuge at 3000rev./min for 10min. The supernatants containing the products of albumin digestion ([^{131}I]iodotyrosine) were assayed for radioactivity.

Gradient centrifugation of the mitochondrial–lysosomal (M+L) fraction

For sucrose-gradient fractionation the livers of rats injected with [1α,2α-^{3}H]cholesterol-labelled liposomes containing ^{131}I-labelled albumin were perfused with 0.9% NaCl and then fractionated in 0.3M-sucrose to obtain the mitochondrial–lysosomal fraction (Gregoriadis & Sourkes, 1967), which was resuspended in 0.3M-sucrose (0.8g of liver/ml). A portion (3ml) of this suspension was layered on the top of a discontinuous sucrose gradient made up as follows: two solutions of 117 and 59.7g of sucrose/100g of water (Beaufay *et al.*, 1964) were mixed in appropriate proportions to give seven solutions of linearly increasing density. Portions (1.5ml) from each of these seven solutions were used for the preparation of the gradient (13.5ml total volume) in 15ml polycarbonate MSE 65 centrifuge tubes. The tubes were centrifuged at 74000$g_{av.}$ for 4.5h in the 6×15ml swinging-bucket rotor of an MSE 65 mark II ultracentrifuge at 4°C.

A Ficoll–mannitol gradient was used for the subfractionation of the M+L fraction obtained from rats injected with β-fructofuranosidase-containing liposomes. Solutions of (*a*) 74g of Ficoll (Beaufay *et al.*, 1964) and 20g of mannitol/100g of water, and (*b*) 20g of mannitol/100g of water, were mixed in appropriate proportions to give eight solutions of linearly increasing density. Portions (1.5ml) from each of these eight solutions were used for the preparation of a discontinuous gradient (12ml total volume) in 15ml polycarbonate centrifuge tubes. A sample (3ml) of the M+L fraction (equivalent to 0.8g of liver/ml) was layered on the top of the gradient. In one experiment, 2.9ml of the M+L fraction from an untreated rat (0.8g of liver/ml) was mixed with 0.1ml of β-fructofuranosidase-containing liposomes and then layered on the top of the gradient. Conditions for centrifugation were identical with those used for the sucrose gradient. At the end of all centrifugations, 1.5ml fractions were carefully pipetted off, starting from the top of the tube.

Enzyme assays

For the measurement of β-fructofuranosidase activity, 0.8ml of a 10% (w/v) sucrose solution in 0.1M-sodium acetate buffer, pH4.5, and 0.1ml of 1% Triton X-100 solution were added to each of two Pyrex tubes and preincubated for a few minutes at 37°C. To one of the tubes 0.1–0.2ml of the sample to be assayed for β-fructofuranosidase activity (liver or spleen homogenates and subcellular fractions) was added, and after 10min at 37°C the tube was heated quickly to the boiling point of its contents. The other tube (which served as control) was also heated and 0.1–0.2ml of the sample to be assayed was added to the boiling solution. Both tubes were cooled at room temperature and 0.02–0.2ml was used for the enzymic measurement of the liberated glucose (Fleming & Pegler, 1963; Catley, 1967). β-Fructofuranosidase in liposomes and blood plasma was measured as total (in the presence of Triton X-100) and as free activity (Triton was added at the end of the 10min incubation and the sample boiled immediately). A unit of β-fructofuranosidase activity is expressed as 1μmol of sucrose hydrolysed/min at 37°C. Assays for the lysosomal marker *N*-acetyl-β-D-glucosaminidase and the mitochondrial marker succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase and expression of their activities have been described previously (Gregoriadis & Ryman, 1972).

Assay of radioactivity

The assay of ^{131}I radioactivity and ^{3}H radioactivity, alone or in the presence of ^{131}I, has been described by Gregoriadis & Ryman (1972).

Results

β-Fructofuranosidase entrapment

Under the conditions described, in four different β-fructofuranosidase-containing liposomal prepara-

tions 11–17mg (2.2–3.4%) of the original β-fructofuranosidase used was entrapped into liposomes (Table 1). β-Fructofuranosidase activity in liposomes was latent (i.e. entrapped) and not bound externally to the liposomal membranes, as most of the enzyme (96–100%) on the day of preparation could be measured only after the disruption of liposomal membranes with Triton X-100 (Table 1). It is noteworthy that the substrate sucrose did not seem to penetrate the liposomal membranes during the assay of the liposomal β-fructofuranosidase in the absence of Triton X-100. If such penetration had occurred, glucose should have been measurable at the end of the assay. Most of the liposomal β-fructofuranosidase was still latent 35 days after its entrapment (Table 1).

Tissue distribution of injected liposomal β-fructofuranosidase activity

After the injection of β-fructofuranosidase-containing liposomes into rats it was possible to record the decline of latent β-fructofuranosidase activity in the blood serum (half-life was attained in about 1h), and its appearance in the liver and spleen (Fig. 1). β-Fructofuranosidase activity in the liver reached a peak at about 6h (45% of the injected dose) and it was

Table 1. *Entrapment of β-fructofuranosidase into liposomes*

β-Fructofuranosidase (500mg) was used for each of four β-fructofuranosidase-containing liposomal preparations, which were kept at 4°C under N_2 for 21 or 35 days. Total β-fructofuranosidase activity in the preparation was measured after disruption of the liposomal membranes with Triton X-100. For free β-fructofuranosidase activity Triton X-100 was omitted (see the Materials and Methods section). Latent β-fructofuranosidase activity (the difference between total and free activity) was expressed as a percentage of the total activity.

β-Fructofuranosidase-containing liposomal preparation no.	Entrapment (% of β-fructofuranosidase used)	Latent β-fructofuranosidase activity (% of the total in preparation)		
		Day 1	Day 21	Day 35
1	2.8	96.0	—	90.0
2	2.2	97.0	—	88.0
3	3.0	100.0	96.0	—
4	3.4	100.0	95.0	—

Table 2. *Intracellular distribution of β-fructofuranosidase activity in the liver of rats injected with β-fructofuranosidase-containing liposomes*

Rats were given 0.5ml of β-fructofuranosidase-containing liposomes (220 units or 1.5mg of β-fructofuranosidase and 17.5mg of lipids), killed at the time-intervals shown and their livers were subfractionated in mannitol medium. In one experiment (rat no. 5, *in vitro*) the liver of an untreated rat was homogenized with 0.3M-mannitol containing 0.1ml of β-fructofuranosidase-containing liposomes (44 units or 0.3mg of β-fructofuranosidase and 3.5mg of lipids) and kept at 4°C for 60min before fractionation. The sum of the β-fructofuranosidase activity in the fractions was 84.3–92.7% of that in the homogenates. Assays of the lysosomal marker *N*-acetyl-β-D-glucosaminidase and the mitochondrial marker succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase were carried out in the homogenates and fractions obtained. As previously reported (Gregoriadis & Ryman, 1972), the mitochondrial–lysosomal fraction had the largest percentage of both enzyme markers. Abbreviations: N, nuclear fraction; M+L, mitochondrial–lysosomal fraction; P, microsomal fraction; S, cytosol.

Rat no.	Time of killing (h)	Activity in homogenate (units/g of liver)	Distribution of activity (% of that in homogenate)			
			N	M+L	P	S
1	0.5	8.8	8.0	43.1	26.5	11.1
2	2.0	18.5	9.4	40.5	24.0	16.2
3	6.0	21.4	7.0	42.5	19.4	15.4
4	21.0	17.6	11.6	34.4	25.1	16.3
5	*in vitro*	40.1	4.5	2.5	72.2	6.0

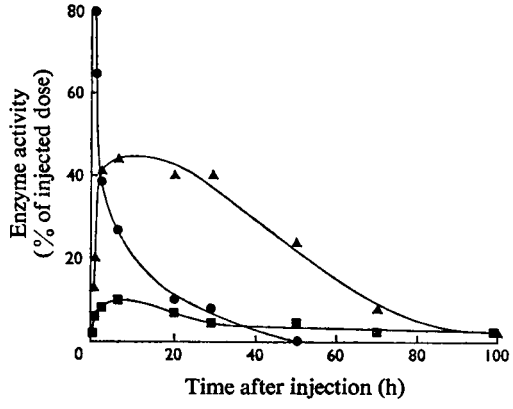


Fig. 1. *β-Fructofuranosidase activity in rat tissues after injection of β-fructofuranosidase-containing liposomes*

Rats were given 0.5ml of β-fructofuranosidase-containing liposomes (220 units or 1.5mg of β-fructofuranosidase and 17.5mg of lipids) and killed at the time-intervals shown. Latent β-fructofuranosidase activity (●) was measured in the blood plasma and total β-fructofuranosidase activity in liver (▲) and spleen (■) and expressed as percentages of the injected activity. No measurable free β-fructofuranosidase activity was found in the blood plasma. The volume of plasma was taken as 4.8ml/100g body wt. (Gregoriadis & Ryman, 1972). Each point represents the average value from two or three rats.

retained at a considerably high value (25%) even after 48h. Similarly, decline of the spleen β-fructofuranosidase activity was very slow (5% of the injected dose by 48h).

Subcellular localization of liposomal β-fructofuranosidase activity

In an earlier investigation of the subcellular fate of ^{3}H in the liver of rats injected with [^{3}H]amyloglucosidase-containing liposomes (Gregoriadis & Ryman, 1972), it was shown that the crude M+L fraction contained the largest proportion of the hepatic radioactivity and it was suggested that amyloglucosidase was associated with the lysosomes. In the present experiments we were able to follow the intracellular distribution of enzyme activity rather than radioactive label, and the results obtained from the liver of rats injected with β-fructofuranosidase-containing liposomes (Table 2) indicate that distribution of β-fructofuranosidase activity favours the M+L fraction up to 21h after injection.

Two attempts were made to investigate further the possibility that liposome-entrapped protein was localized in the hepatic lysosomes of rats injected with such protein-containing liposomes.

Incubation of the liver M+L fraction

The lysosome-rich M+L fraction of the liver of rats injected with ^{131}I-labelled albumin-containing liposomes was suspended in osmotically protected medium and incubated at 22°C (see the Materials and Methods section). It was expected that if ^{131}I-labelled albumin was localized in lysosomes it would be subjected to the attack of lysosomal enzymes with the subsequent release of [^{131}I]iodotyrosine (Mego *et al.*, 1967). At various time-intervals samples of the M+L suspension were mixed with trichloroacetic acid to precipitate the proteins (including non-digested ^{131}I-labelled albumin) and the supernatants containing acid-soluble digestion products of ^{131}I-labelled albumin were assayed for radioactivity (Fig. 2). In five separate experiments, after a 2h incubation period the original percentage of acid-soluble radioactivity in the M+L fraction approximately doubled. In a typical experiment (Fig. 2) there was an increase in acid-soluble radioactivity from 9.5% (of the total radioactivity) to 17%. In contrast, incubation of the same M+L fraction, previously treated with Triton X-100 (which disrupted the lysosomes and caused dilution of the lysosomal enzymes), did not result in any increase in acid-soluble radioactivity. Similarly, no increase in acid-soluble radioactivity was observed for the M+L fraction, from an untreated rat, mixed *in vitro* with liposomes containing ^{131}I-labelled albumin (Fig. 2).

Sucrose-gradient fractionation of the liver M+L fraction

Results obtained on fractionation of the M+L fractions of the livers of rats injected with [1α,2α-^{3}H]-cholesterol-labelled liposomes containing ^{131}I-labelled albumin are shown in Fig. 3. Both liposomal radioactive labels were recovered on the top of the gradient [^{3}H:(*a*);^{131}I:(*b*)], as opposed to the lysosomal (*c*) and mitochondrial (*d*) markers, which were recovered in 'heavier' areas of the gradient.

Ficoll–mannitol-gradient fractionation of the liver M+L fraction

The property of Triton WR-1339 and dextran, when injected into rats, respectively to decrease (Wattiaux *et al.*, 1963) or increase (Baudhuin *et al.*, 1965) the specific gravity of liver lysosomes was utilized. To investigate possible lysosomal localization of liposomal β-fructofuranosidase, rats were injected with 0.9% NaCl, Triton WR-1339 or dextran 3.5 days before the administration of β-fructofuranosidase-containing liposomes, and the M+L fraction of their livers, after removal of blood, was then subfractionated on a Ficoll–mannitol discontinuous gradient. β-Fructofuranosidase activity, the lysosomal marker *N*-acetyl-β-D-glucosaminidase and the

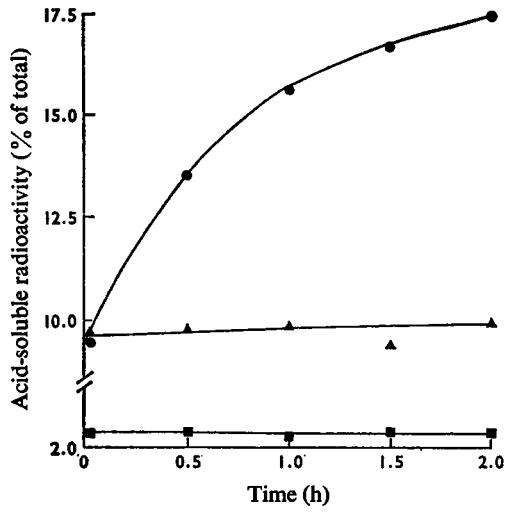


Fig. 2. *Incubation of the mitochondrial–lysosomal fraction of the liver of rats injected with liposomes containing ^{131}I-labelled albumin*

Five rats were injected with 0.5ml of liposomes containing ^{131}I-labelled albumin [3mg of albumin (3×10^5c.p.m.) and 17mg of lipid] and killed after 30min. Suspensions of their liver mitochondrial–lysosomal fraction were prepared in 0.1M-tris–HCl buffer, pH7.4, containing 0.3M-sucrose (5ml/g of liver) and each suspension was divided into two equal parts. To one part Triton X-100 was added (final concn. 0.1%) and, to the other, buffered sucrose. Both suspensions were incubated at 22°C for 2h (see the Materials and Methods section). A mitochondrial–lysosomal suspension (with no Triton X-100 added) from an untreated rat was mixed with 0.1ml of liposomes containing ^{131}I-labelled albumin [0.6mg of albumin (6×10^4c.p.m.) and 3.4mg of lipid] and incubated as described above. At the time-intervals shown samples were tested for trichloroacetic acid-soluble radioactivity, which, in a typical experiment shown here, is expressed as a percentage of the total in the sample (range of total radioactivity per sample in all five experiments was 10^4–1.5×10^4c.p.m.). ●, Mitochondrial–lysosomal fraction from a rat injected with albumin-containing liposomes; ▲, same fraction with Triton X-100 added; ■, mitochondrial–lysosomal fraction from an untreated rat mixed *in vitro* with albumin-containing liposomes.

mitochondrial marker succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase were measured in the subfractions obtained. It was expected that if β-fructofuranosidase was localized in the lysosomes, an identical displacement of β-fructofuranosidase and of the lysosomal marker upwards (lysosomes laden with Triton WR-1339) and downwards (lysosomes laden with dextran) on the gradient would be observed.

In rats that received no treatment (0.9% NaCl) before the injection of β-fructofuranosidase-containing liposomes, β-fructofuranosidase activity in the gradient profile (Fig. 4*a*) exhibited a major peak (68% of the total) in the area of the gradient of density 1.188–1.235g·cm^{-3} ('heavy' area) and a minor one (18%) in the area of the gradient of density 1.081–1.125g·cm^{-3} ('light' area). A similar pattern was obtained for *N*-acetyl-β-D-glucosaminidase (81% in 'heavy' area, 12% in 'light' area) and succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase (90% in 'heavy' area, 16% in 'light' area).

Treatment of rats with Triton WR-1339 before the injection of β-fructofuranosidase-containing liposomes resulted in a shift of β-fructofuranosidase activity from the 'heavy' area of the gradient to the 'light' area. Thus 50% of the activity was found in the 'light' area and only 38% in the 'heavy' area (Fig. 4*b*). There was a similar shift of the lysosomal marker towards the lighter area of the gradient (37% in the 'heavy' area, 41% in the 'light' area; Fig. 4*b*). There was no change in the pattern of the mitochondrial marker (Fig. 4*b*).

The displacement of β-fructofuranosidase activity towards the 'light' area of the gradient in Triton WR-1339-treated rats could, however, be an artifact. Triton WR-1339 is a non-ionic detergent and, although a mild one, residual amounts of this compound in the blood 3.5 days after its injection into rats could probably disrupt injected liposomes and liberate β-fructofuranosidase into the circulation. In such a case, free β-fructofuranosidase would have been taken up by the liver and stored in the lysosomes (Jacques & Bruns, 1965), thus giving the profile shown in Fig. 4(*b*). This possibility was excluded by mixing 2ml of heparin-treated rat blood with 20mg of Triton WR-1339 and 0.1ml of β-fructofuranosidase-containing liposomes (preparation 4, Table 1) and stirring gently at 37°C. No free β-fructofuranosidase was found in samples of blood plasma after 3h incubation.

Administration of dextran to rats before injection of β-fructofuranosidase-containing liposomes resulted in a slight redistribution of β-fructofuranosidase activity, favouring the 'heavy' area ('light' area, 11%, 'heavy' area, 72%; Fig. 4*c*). The effect of dextran in increasing the specific gravity of lysosomes was more obvious, however, within the 'heavy' area: most of the β-fructofuranosidase activity was displaced to the bottom of the tube (Fig. 4*c*). A similar displacement of activity to the bottom of the tube was also observed with *N*-acetyl-β-D-glucosaminidase but not with the mitochondrial marker (Fig. 4*c*).

Finally, mixing *in vitro* of the mitochondrial–lysosomal fraction of an untreated rat with β-fructofuranosidase-containing liposomes did not result in any association of liposomes with lysosomes or mitochondria. Enzyme patterns (Fig. 4*d*) showed that, although most of the β-fructofuranosidase activity (77%) was recovered in the 'light' area, lysosomal and mitochondrial markers were distributed normally, as in Fig. 4(*a*).

Discussion

Brady (1971) has outlined known possibilities for the treatment of storage diseases in which a specific enzyme activity is absent from one or more tissues. Enzyme-replacement therapy in the form of direct parenteral administration of enzymes to patients has been attempted in the past (Baudhuin *et al.*, 1964; Fernandes & Huijing, 1968; Kissel *et al.*, 1968). However, a free foreign enzyme injected into the circulation can provoke immunological and other systemic reactions and it eventually reaches the reticulo-endothelial system. As an alternative, encapsulation of enzymes into synthetic porous microspherules, through which substrates from the environment could diffuse freely, has been proposed by Chang (1966). His technique, however, is at present limited to cases in which excess of substrate of low molecular weight accumulates in the body fluids.

Owing to our interest in lysosomal storage diseases we have attempted to develop a system by which enzymes designed to remove undesirable accumulated products can be carried to specific tissues of the human body. We thought liposomes to be suitable candidates for enzyme entrapment because they are made of physiological materials (phosphatidylcholine, cholesterol, phosphatidic acid), they are biodegradable and their surfaces can be chemically manipulated to our specifications.

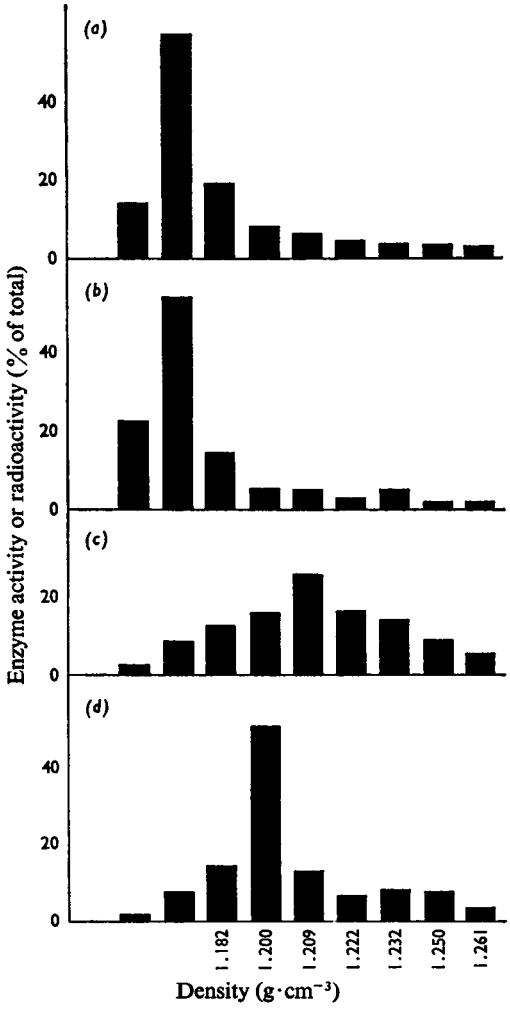
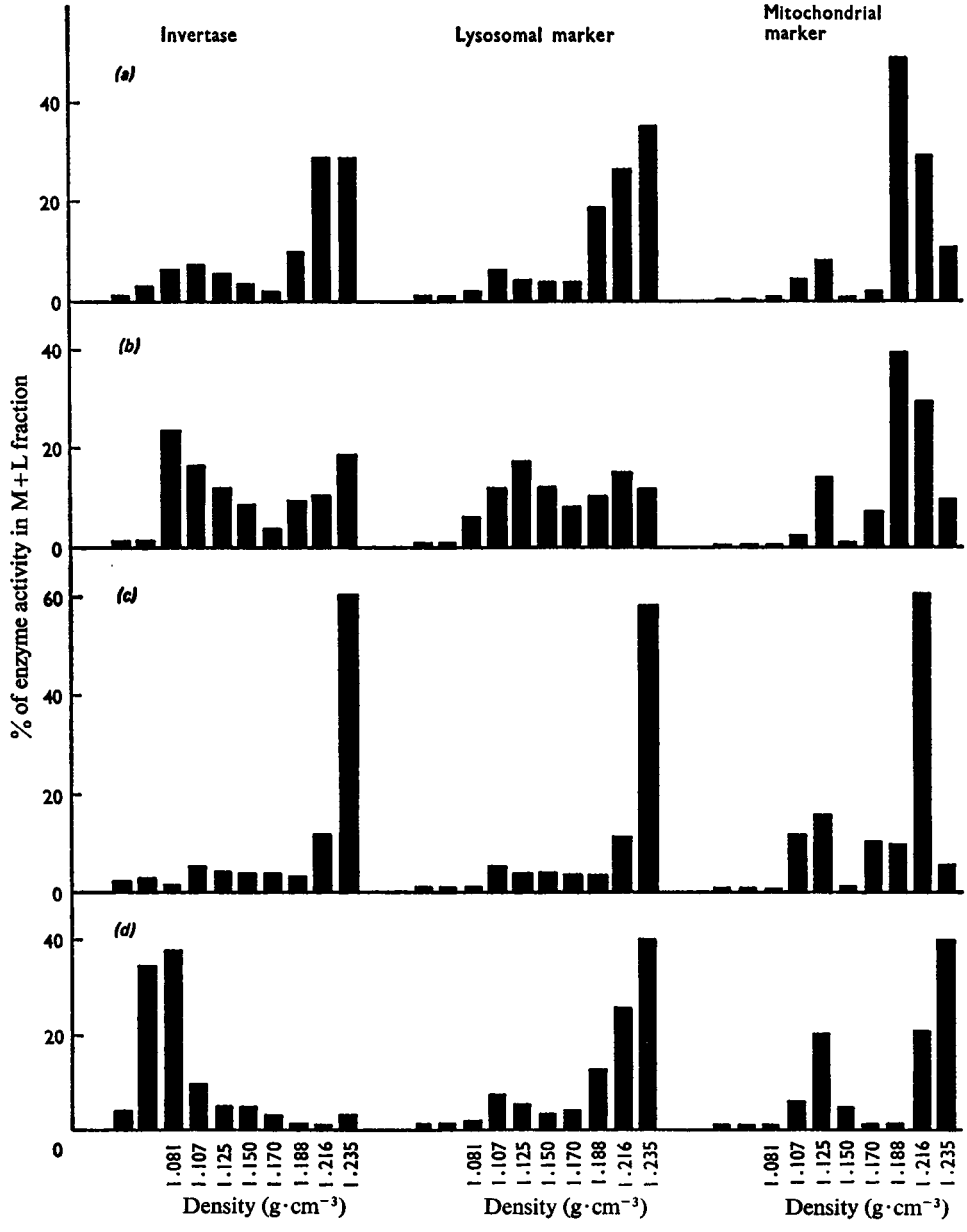


Fig. 3. *Gradient fractionation of the mitochondrial–lysosomal fraction of the liver of rats injected with* [1α,2α-^3H]*cholesterol-labelled liposomes containing* ^{131}I*-labelled albumin*

Rats were injected with 1ml of [1α,2α-^{3}H]cholesterol-labelled liposomes containing ^{131}I-labelled albumin [0.2mg of albumin (8.7×10^5c.p.m.) and 3.6mg of lipid (3.6×10^6d.p.m.)] and killed after 30min. A sample (3ml) of the mitochondrial–lysosomal fraction of the liver (2.7×10^4c.p.m. for ^{131}I and 1.4×10^5d.p.m. for ^{3}H) was subfractionated in a sucrose gradient (see the Materials and Methods section) and ^{131}I (*a*), ^{3}H (*b*), *N*-acetyl-β-D-glucosaminidase (*c*) and succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium reductase (*d*) were assayed in the nine subfractions obtained. Each bar (average value from two rats) represents enzyme activity or radioactivity as a percentage of the total recovered in the nine fractions. The sum of each of the two radioactivities and two enzyme activities respectively was 90–107% of the radioactivity or enzyme activity measured in the original volume of the mitochondrial–lysosomal fraction used for gradient fractionation. The density of the seven sucrose solutions used for the preparation of the gradient (see the Materials and Methods section) was measured as described by Gregoriadis *et al.* (1970). The first two fractions, the total volume of which corresponds to that of the layered M+L suspension, fall beyond the limits of the gradient.

Our experiments demonstrated that a considerable amount of enzyme (11–17 mg/preparation; 2.2–3.4% of the starting amount of β-fructofuranosidase) can be entrapped into liposomes, which can then be separated from the non-entrapped enzyme (Table 1). Enzyme activity was almost totally absent from the surface of liposomes containing amyloglucosidase or β-fructofuranosidase (Gregoriadis *et al.*, 1971; and Table 1). Treatment with Triton X-100 liberated most of the enzyme activity. No leaking of the protein contents of liposomes was observed in the blood of rats injected with liposomes containing ^{131}I-labelled albumin (Gregoriadis & Ryman, 1972). In rats injected with β-fructofuranosidase-containing liposomes, all β-fructofuranosidase activity in the blood was latent and much of it was eventually recovered



in the liver and to a lesser extent in the spleen (Fig. 1). Uptake of liposomes by the spleen and the finding that saturation of the reticulo-endothelial system with carbon particles before the administration of liposomes containing ^{131}I-labelled albumin results in a considerable (about 25%) decline in the hepatic uptake of liposomes (G. Gregoriadis & B. E. Ryman, unpublished work) strongly suggest that, in addition to the parenchymal cells of the liver (Gregoriadis & Ryman, 1972), the reticulo-endothelial system might participate to a limited extent in liposomal uptake.

β-Fructofuranosidase activity in the liver of rats injected with β-fructofuranosidase-containing liposomes slowly declines from a maximum of 45% of the injected dose at 6h to 5% in 100h (Fig. 1). This decrease in activity, also observed in the spleen, implies catabolism of the enzyme, presumably after rupture of liposomal membranes. Such enzyme catabolism in fact has been demonstrated in rats treated with [^{3}H]amyloglucosidase-containing liposomes (Gregoriadis & Ryman, 1972); mixing of the liver homogenates of such animals with rabbit anti-(amyloglucosidase) serum showed a gradual fall in the immunoprecipitable ^{3}H/total ^{3}H ratio with time.

Subcellular-fractionation studies of the liver of animals injected with liposomes containing [^{3}H]-amyloglucosidase or β-fructofuranosidase indicated that ^{3}H (Gregoriadis & Ryman, 1972) or β-fructofuranosidase activity (Table 2) was primarily localized in the mitochondrial–lysosomal fraction. This and the observed catabolism of [^{3}H]amyloglucosidase strongly favoured a lysosomal localization of liposomes.

The answer to the question whether a protein carried by liposomes to the liver is eventually localized in the lysosomes was attempted in two fashions: Mego *et al.* (1967) have shown that denatured ^{131}I-labelled albumin, when intravenously injected into mice, is taken up into the liver lysosome-rich fraction. Incubation of this fraction in osmotically protected medium resulted in further degradation of the labelled albumin with release of [^{131}I]iodotyrosine. Release of labelled tyrosine in the incubation medium was considered by Mego *et al.* (1967) to be an indication of lysosomal catabolism of the injected labelled albumin. Similar findings with rats have been reported by Davies *et al.* (1969). The present incubation studies of the lysosome-rich fraction of the liver of rats injected with liposomes containing ^{131}I-labelled albumin (Fig. 2) demonstrate catabolism of ^{131}I-labelled albumin, presumably within the lysosomes, this being a continuation of liposomal albumin catabolism carried out *in vivo*.

Catabolism of a liposomal protein by the liver of animals injected with protein-containing liposomes implies that such catabolism occurs in the liver lysosomes. It is necessary, however, to demonstrate that liposomal protein is actually localized within the lysosomes. This was attempted by gradient fractionation experiments. Sucrose-gradient fractionation studies with the M+L fraction from the liver of rats injected with [1α,2α-^{3}H]cholesterol-labelled liposomes containing ^{131}I-labelled albumin (Fig. 3) indicated that although the two liposomal radioactive labels (^{131}I and ^{3}H) sedimented similarly, they were recovered on the top of the gradient (density $< 1.182\,g\cdot cm^{-3}$), and they were clearly separated from the lysosomal and mitochondrial markers, which were recovered in bulk at an area of the gradient with a density of 1.182–1.222 (Fig. 3). We thought of two possible reasons for such a pattern of liposome

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Fig. 4. *Gradient fractionation of the mitochondrial–lysosomal fraction of the liver of rats injected with β-fructofuranosidase-containing liposomes*

Rats were injected with 0.5ml of β-fructofuranosidase-containing liposomes (250 units or 1.7mg of β-fructofuranosidase and 18.0mg of lipids) 3.5 days after injection of 1.0ml of 0.9% NaCl (*a*), 200mg of Triton WR-1339 (*b*) or 200mg of dextran (*c*) and killed after 2h. Mitochondrial–lysosomal fraction of the liver (3ml, equivalent to 0.8g of liver/ml) containing 20–27 units of β-fructofuranosidase was subfractionated in a Ficoll–mannitol gradient (see the Materials and Methods section). In one experiment 2.9ml of the mitochondrial–lysosomal fraction of an untreated rat was mixed with 0.1ml of β-fructofuranosidase-containing liposomes (50 units of β-fructofuranosidase and 3.6mg of lipids), kept at 4°C for 60min and then subfractionated as described above (*d*). β-Fructofuranosidase, *N*-acetyl-β-D-glucosaminidase (lysosomal marker) and succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium reductase (mitochondrial marker) were assayed in the ten subfractions obtained. Each bar (average value from two rats) represents enzyme activity as a percentage of the total recovered in the ten fractions. The sum of each of the three enzyme activities in all fractions was 93–115% of the activity measured in the original volume of the mitochondrial–lysosomal fraction used for gradient fractionation. The density of the eight Ficoll–mannitol solutions used for the preparation of the gradient (see the Materials and Methods section) was measured pycnometrically. The first two fractions, the total volume of which corresponds to that of the layered M+L suspension, fall beyond the limits of the gradient.

sedimentation: either (*a*) liposomes in the M+L fraction were free (not incorporated into cell organelles), or (*b*) liposomes were incorporated into cell organelles, which, owing to the fatty composition of liposomes, had their specific gravity decreased. The first possibility has been discussed elsewhere (Gregoriadis & Ryman, 1972) and shown to be incompatible with a variety of experimental results. On the other hand, lysosomes loaded with Triton WR-1339 are known to become lighter (Wattiaux *et al.*, 1963) and it is possible that some of the lysosomes loaded with liposomal lipids attain a decreased specific gravity.

Replacement of the sucrose gradient with a Ficoll–mannitol gradient in the fractionation of the liver M+L fraction from rats injected with β-fructofuranosidase-containing liposomes resulted in an enzyme pattern in which β-fructofuranosidase, lysosomal marker and mitochondrial marker are recovered in the same range of gradient density (Fig. 4*a*). The exhibition by all three enzymes of a major peak at a gradient density of 1.188–1.235 and a minor one at a density of 1.081–1.125 $g \cdot cm^{-3}$ suggests that β-fructofuranosidase is associated with lysosomes or mitochondria. Again, the possibility that free liposomes, entrapped in extracellular spaces of the liver, sedimented similarly to lysosomes and mitochondria was rejected. The profiles of the lysosomal and mitochondrial markers and β-fructofuranosidase, after mixing *in vitro* of β-fructofuranosidase-containing liposomes with the mitochondrial lysosomal fraction of the liver of untreated rats before gradient fractionation, were dissimilar (Fig. 4*d*). Thus, although both marker profiles were identical with those in Fig. 4(*a*), β-fructofuranosidase activity was recovered quantitatively on the top of the gradient.

To investigate further whether β-fructofuranosidase is associated with lysosomes, we utilized the property of Triton WR-1339 (Wattiaux *et al.*, 1963) and dextran (Baudhuin *et al.*, 1965) to alter the specific gravity of lysosomes *in vivo*. The technique used (see the Materials and Methods section) has been employed in the past for a similar purpose, i.e. to demonstrate the lysosomal localization of desialylated caeruloplasmin (Gregoriadis *et al.*, 1970) which similarly to lysosomes, when injected into rats, is taken up by the hepatic parenchymal cells. (Gregoriadis *et al.*, 1970). In experiments with rats injected with Triton WR-1339 or dextran 3.5 days before the injection of β-fructofuranosidase-containing liposomes, there was a parallel displacement of β-fructofuranosidase and the lysosomal marker upwards (Fig. 4*b*) and downwards (Fig. 4*c*) on the gradient. These results indicate lysosomal localization of β-fructofuranosidase.

It is noteworthy that the displacement of β-fructofuranosidase was more pronounced than that of the lysosomal marker (in Triton-WR-1339-treated rats) and it is possible that this was the result of an accumulative effect of Triton WR-1339 and liposomal lipids in decreasing the lysosomal specific gravity.

Experiments described in the present paper and elsewhere (Gregoriadis & Ryman, 1972) appear to indicate that liposomes can deliver enzymes into the lysosomes of the cells of the liver (parenchymal and probably Kupffer) and spleen. However, such cells need not be the only target for liposomes. Modifications of the liposomal membrane could possibly induce specific endocytosis in various cell types and tissues of the body.

Assuming that liposome-carried enzymes can be active within the lysosomes for long enough to degrade unwanted material, then enzyme- (or indeed drug-) containing liposomes must be seriously considered for the treatment of storage diseases. It may also prove possible to use liposomes in the treatment of other diseases, among which cancer is an obvious example. One could envisage at least two approaches in the utilization of liposomes in cancer chemotherapy: (*a*) liposome-entrapped antitumour drugs (e.g. 5-fluorouracil) could be delivered to the lysosomes of the tissue and then, after rupture of liposomal membranes, hopefully diffuse through the lysosomal membranes, the permeability of which, if necessary, could be altered by a liposome-entrapped agent; (*b*) agents that release and thereby activate lysosomal hydrolases (e.g. vitamin A), and which subsequently bring about degradative changes in the tumour cells, have been suggested as an approach to cancer chemotherapy (de Duve, 1968; Allison, 1969). Such agents could be entrapped into liposomes and delivered directly into the lysosomes of diseased tissues. Drugs entrapped into liposomes or incorporated into the liposomal membranes will have the advantage of being therapeutic in small doses (owing to selective uptake of liposomes) and of lacking the side effects that usually accompany direct drug administration. Further, drug resistance arising in some cases (Bosmann, 1971) through modification of cell-membrane permeability to the drug might not develop.

Administration of messenger RNA or DNA, or hybridization and replacement of the patient's cells (genetic engineering), appears to be the ultimate therapeutic approach for at least some of the genetic disorders, and the need for such an approach seems well established (Tatum, 1966). The possibility of employing liposomes to introduce segments of genetic material to defective cells, coupled with modification of relevant lysosomal enzyme activities or lysosomal membrane permeability or both, is worth considering.

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