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Rapid Partial Purification of Placental Glucocerebroside β-Glucosidase and its Entrapment in Liposomes

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1. A glucocerebroside β-glucosidase-rich detergent-free preparation was obtained from human placentas by a rapid method combining affinity chromatography on concanavalin A–Sepharose and organic-solvent precipitation. In a typical preparation about 11 000 units of the enzyme purified 1500-fold were obtained from five placentas in 2 days. 2. The enzyme preparation also contained other hydrolases, but the extent of their purification was much smaller. 3. Studies on entrapment in liposomes showed that all glucocerebroside β-glucosidase activity used could be incorporated in neutral egg phosphatidylcholine–cholesterol liposomes. Association with liposomes appeared to discriminate against other proteins, including some of the hydrolases, thus contributing to further purification of the enzyme. More than 95% of the liposome-associated enzyme activity was latent.

Enzyme-replacement therapy for storage diseases is associated with a number of difficulties. For instance, the enzyme may fail to reach sites in need of treatment or it may be inactivated by immunological or other factors (Hers, 1973; Brady & King, 1973). Gregoriadis (1976*a*,*b*) has shown that the use of liposomes as enzyme carriers can prevent the enzyme from acting on non-target substrates in the blood (Gregoriadis *et al.*, 1974), protect the enzyme from its circulating antibodies (Neerunjun & Gregoriadis, 1976) and provide a mechanism for its direction to areas where it is needed (Gregoriadis & Neerunjun, 1975; Weissmann *et al.*, 1975; Juliano & Stamp, 1976). Since injected liposomes appear to transport enzymes into the lysosomes of the liver and spleen cells, predominantly those of the reticuloendothelial system (Segal *et al.*, 1974; Rahman & Wright, 1975; Wisse *et al.*, 1976), lysosomal storage disorders involving such tissues are relevant to the liposome approach. Indeed, enzyme-containing liposomes have been used successfully for the correction of model lysosomal-storage conditions (Gregoriadis & Buckland, 1973; Colley & Ryman, 1974; Roerdink *et al.*, 1976). An attractive candidate for enzyme therapy via liposomes is adult Gaucher's disease, in which, because of a deficiency in the lysosomal glucocerebroside β-glucosidase, the glycosphingolipid glucocerebroside accumulates in the lysosomes of the liver and spleen (Brady *et al.*, 1965; Patrick, 1965). However, purification of glucocerebroside β-glucosidase from human placenta is lengthy and also difficult to apply to a large-scale production (Pentchev *et al.*, 1973), so that unavailability of the enzyme is a major obstacle for its use in treatment.

In the hope that liposomes will provide us with a means for the controlled and safe release of glucocerebroside β-glucosidase in the diseased tissues of adult patients with Gaucher's disease we have, as a prelude to treatment, developed a method for the rapid preparation of the enzyme, in a form suitable for entrapment in liposomes, from large amounts of human placental tissue. Preliminary results of this work have been presented elsewhere (Braidman & Gregoriadis, 1976).

Materials and Methods

Human placentas were obtained from the maternity ward, Northwick Park Hospital, Harrow, Middx., U.K. Human spleen glucocerebroside and glucocerebroside *N*-acylated with [^{14}C]stearic acid were kindly supplied by Dr. A. D. Patrick, Institute of Child Health, London W.C.1, U.K., and Dr. Mae Wan Ho, Department of Biochemistry, Queen Elizabeth College, London W8 7AN, U.K., respectively. The labelled glucocerebroside was further purified by preparative t.l.c. (Kopaczyk & Radin, 1965). The sources and grades of egg phosphatidylcholine, cholesterol, phosphatidic acid, dicetyl phosphate and stearylamine have been described elsewhere (Gregoriadis & Neerunjun, 1974). 4-Methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside, 4-methylumbelliferyl α-mannoside, 4-methylumbelliferyl α-D-glucoside and 4-methylumbelliferyl α-D-galactoside were supplied by Koch–Light Laboratories, Colnbrook, Bucks., U.K.; 4-methylumbelliferyl β-D-glucoside, α-methyl D-mannoside, human γ-globulin (Cohn fraction II), horseradish peroxidase (type I) and cytochrome *c* (horse heart, type II) were from Sigma (London)

Chemical Co., London S.W.6, U.K.; Triton X-100 (grade A) and sodium taurocholate (grade B) were from Calbiochem, San Diego, CA, U.S.A.; Triton WR1339 was a gift from Winthrop Laboratories Ltd., Fawdon, Newcastle upon Tyne, U.K.; concanavalin A–Sepharose and Blue Dextran 2000 were from Pharmacia, London W.5, U.K.; poly(ethylene glycol) 6000 and bovine serum albumin were from British Drug Houses, Poole, Dorset, U.K. All other reagents were of analytical grade.

Preparation of glucocerebroside β-glucosidase

Human placentas (300–600g each) kept at 4°C were used within 24h of delivery, and in the procedure described all steps were carried out at 4°C unless otherwise stated. In a typical preparation, five placentas were freed of chorion membranes, washed with water, cut into small pieces with a stainless-steel knife and minced through a meat grinder. The pulp obtained was then mixed with 10mM-sodium phosphate buffer, pH7.0 (30%, w/v), homogenized in a Waring blender for 5min and then centrifuged in an MSE 18 centrifuge at 23000*g* in a continuous-flow head. The pellet was rehomogenized in 10mM-sodium phosphate buffer, pH7.0 (30%, w/v), containing 0.15% (v/v) Triton WR1339 and centrifuged as above. The supernatant was centrifuged again in an MSE 18 centrifuge for 30min at 26000*g* ($r_{av.}$ 14.4cm) in an angle head to obtain a clear solution. The clear supernatant (usually 900–1000ml) was applied to 100–200ml of concanavalin A–Sepharose held in a sintered-glass funnel (9cm height × 15cm diameter) and equilibrated with 10mM-sodium phosphate buffer, pH7.0, containing 0.15% Triton WR1339. Non-adsorbed material was washed exhaustively with the same buffer and bound proteins were eluted at room temperature (16°C) with 300ml of 10mM-sodium phosphate buffer, pH7.0, containing 0.15% Triton WR1339, 1M-α-methyl D-mannoside and 0.67M-EDTA. Concanavalin A–Sepharose was regenerated with 10mM-sodium phosphate buffer, pH7.0, containing 0.15% Triton WR1339, 0.1mM-$MnCl_2$, 0.1mM-$CaCl_2$ and 0.25% (w/v) concanavalin A. The eluate containing the enzyme together with other proteins was mixed with 4vol. of ethanol/chloroform (9:1, v/v), left at 4°C for 2h and then centrifuged for 30min at 26000*g* ($r_{av.}$ 14.4cm). The precipitate was washed 2–3 times with about 100ml of the solvent mixture to remove excess of detergent, and after the final centrifugation (26000*g* for 30min) it was flushed with N_2 to eliminate the solvents and subsequently redissolved in 10ml of 10mM-sodium phosphate buffer, pH7.0. The solution was then dialysed against the same buffer to remove traces of α-methyl D-mannoside or organic solvents and concentrated to an appropriate volume (2–7ml/kg of placenta) by dialysis against poly(ethylene glycol) 6000 for 2h. The solution, which was rich in glucocerebroside β-glucosidase and other hydrolases, was used immediately or after overnight storage at 4°C.

Entrapment of glucocerebroside β-glucosidase in liposomes

Entrapment of glucocerebroside β-glucosidase in neutral, negatively and positively charged liposomes was carried out by a general procedure described by Gregoriadis (1976*c*). In brief, 40μmol of egg phosphatidylcholine and 11.4μmol of cholesterol (neutral) or the same supplemented with 5.7μmol of dicetyl phosphate (negative), with 5.7, 11.4 or 22.4μmol of phosphatidic acid (negative) or with 5.7μmol of stearylamine (positive liposomes) were dissolved in chloroform, which was subsequently eliminated by rotary evaporation under reduced argon pressure. The lipid film was flushed with argon to eliminate traces of chloroform, and subsequently disrupted in the presence of four or five glass beads with 2ml of the enzyme solution (containing β-glucosidase and other hydrolases) to form liposomes and left at 4°C. Then 1–2h later the suspension was centrifuged in an MSE Superspeed 65 centrifuge for 60min at 100000*g* ($r_{av.}$ 5.78cm) and the liposomal pellet, which contained the β-glucosidase together with other enzymes, was suspended in 1ml of 10mM-sodium phosphate buffer, pH7.0, and kept under argon at 4°C.

Enzyme assays

Assay of β-glucosidase (EC 3.2.1.21) activity and determination of pH–activity profiles were carried out with both natural substrate, radiolabelled glucocerebroside diluted with non-radioactive glucocerebroside to a specific radioactivity of about 5000c.p.m./nmol, and synthetic substrate, 4-methylumbelliferyl β-D-glucoside as described by Ho *et al.* (1973), with the inclusion of 0.1% (w/v) sodium taurocholate in the reaction mixture (Ho, 1973). *N*-Acetyl-β-glucosaminidase (EC 3.2.1.30), α-mannosidase (EC 3.2.1.24) α-glucosidase (EC 3.2.1.20) and α-galactosidase (EC 3.2.1.22) were determined by the method of Robinson *et al.* (1972), modified as follows: 50μl of the enzyme sample in duplicate was incubated with an equal volume of 2.0mM of the appropriate substrate dissolved in sodium phosphate/citric acid buffer, pH4.5 (McIlvaine, 1921). After 5 or 10min at 37°C the reaction was terminated by the addition of 2.0ml of 0.05M-glycine adjusted to pH10.4 with 0.2M-NaOH. The 4-methylumbelliferone released was measured fluorimetrically in a Perkin–Elmer spectrofluorimeter. To estimate the extent of entrapment of glucosidase and of other hydrolases in liposomes, the liposome suspension before centrifugation at 100000*g* for 60min, $r_{av.}$ 5.78cm (total activity), the suspended liposomal pellet after centrifugation (entrapped activity) and the supernatant (non-

entrapped activity) were mixed with Triton X-100 (final concn. 0.8%) and left for 10min at 4°C. Enzyme activity was then measured as above in 50μl duplicate samples. Latency of the entrapped activity in the suspended liposomal pellet was measured by assaying the enzyme in the presence and in the absence of Triton X-100. All enzyme units are expressed as nmol of substrate hydrolysed at 37°C/min.

Protein determination

Protein in the various fractions in the enzyme-purification procedure and in all other experiments described was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Protein measurement in liposomes was carried out after these were disrupted with Triton X-100.

Polyacrylamide-gel electrophoresis

Electrophoresis of β-glucosidase-rich preparations on polyacrylamide gels was carried out for 1h (Davis, 1964). Protein bands were detected by staining with either Amido Black or Coomassie Blue. For the measurement of β-glucosidase activity, one of several gels run concurrently was kept at −20°C overnight immediately after electrophoresis and subsequently cut into 1.5mm slices. These were placed individually in 0.5ml of 3mM-4-methylumbelliferyl β-D-glucoside containing 0.1% sodium taurocholate and 0.1% Triton X-100 and incubated at 37°C for 60min. The reaction was stopped with 2ml of 0.05M-glycine buffer adjusted to pH10.4 with 0.2M-NaOH. Protein content in individual gel slices was measured after their solubilization for 2h in tubes containing 2ml of 20% (v/v) H_2O_2 and kept in boiling water.

Gel filtration

For the approximate estimation of the molecular weight of β-glucosidase in the final enzyme preparation, 700 units of β-glucosidase were applied to a Sephadex G-200 column (40cm×2.5cm) equilibrated with 10mM-sodium phosphate buffer, pH7.0, containing 0.15% Triton X-100 and 0.1% sodium taurocholate. γ-Globulin (10mg), bovine serum albumin (10mg), horseradish peroxidase (175 units) and cytochrome *c* (5mg) were used to calibrate the column (Andrews, 1965) and the void volume (V_0) was determined by applying Blue Dextran to the column.

Table 1. *Purification of glucocerebroside β-glucosidase from human placenta*

For the assay of glucocerebroside β-glucosidase activity the ^{14}C-labelled glucocerebroside was used as substrate. All other enzyme activities were measured with the appropriate 4-methylumbelliferyl glycoside. Initial activities in the crude placental homogenate (430g of total protein) were as follows: *N*-acetyl-β-glucosaminidase, 18.90×10^6; α-mannosidase, 2.12×10^6; α-glucosidase, 4.50×10^6; α-galactosidase, 0.70×10^6; β-glucosidase, 1.40×10^6; glucocerebroside β-glucosidase, 0.76×10^6 units.

Step of purification	Enzyme	Specific activity (units/mg of protein)	Purification (fold)	Yield (% of total initial activity)
1. Homogenization of placenta in 10mM-sodium phosphate buffer, pH7.0	*N*-Acetyl-β-glucosaminidase	44.0	—	—
	α-Mannosidase	4.9	—	—
	α-Glucosidase	10.5	—	—
	α-Galactosidase	1.6	—	—
	β-Glucosidase	3.2	—	—
	Glucocerebroside β-glucosidase	1.8	—	—
2. Extraction with 10mM-sodium phosphate buffer containing Triton WR1339	*N*-Acetyl-β-glucosaminidase	15.1	0.3	3.9
	α-Mannosidase	2.8	0.6	6.6
	α-Glucosidase	5.3	0.5	5.7
	α-Galactosidase	0.9	0.6	6.2
	β-Glucosidase	2.6	0.8	9.3
	Glucocerebroside β-glucosidase	5.1	2.8	32.8
3. Elution of concanavalin A–Sepharose with 1M-α-methyl D-mannoside and 0.67M-EDTA in 10mM-sodium phosphate buffer	*N*-Acetyl-β-glucosaminidase	1573.3	35.7	3.1
	α-Mannosidase	320.0	65.3	5.6
	α-Glucosidase	666.6	63.4	5.5
	α-Galactosidase	106.6	66.6	5.7
	β-Glucosidase	373.3	116.6	10.0
	Glucocerebroside β-glucosidase	693.3	385.1	34.2
4. Precipitation with ethanol/chloroform (9:1, v/v)	*N*-Acetyl-β-glucosaminidase	2200.0	50.0	0.1
	α-Mannosidase	900.0	183.6	0.2
	α-Glucosidase	1576.0	150.0	0.1
	α-Galactosidase	275.0	171.8	0.2
	β-Glucosidase	1125.0	351.5	0.3
	Glucocerebroside β-glucosidase	2750.0	1528.0	1.4

Results

Purification of glucocerebroside β-glucosidase

The whole procedure of glucocerebroside β-glucoside purification, usually involving 1.6–2.2kg of fresh placenta, took 2 days to complete, and, although the yield of activity varied, in a typical preparation about 1.5% of the original enzyme activity in the crude placental homogenate was recovered after the final stage of the purification procedure. This corresponded to more than 1500-fold purification when enzyme assays were performed with the natural substrate (Table 1). In contrast, the yield was one-quarter to one-fifth of this (0.32%; Table 1) when the synthetic substrate was used. Four other hydrolases (i.e. *N*-acetyl-β-glucosaminidase, α-mannosidase, α-glucosidase and α-galactosidase) were also purified, although to lesser degrees, and yield, compared with that of glucocerebroside β-glucosidase, was much smaller (Table 1). This was especially true for *N*-acetyl-β-glucosaminidase, of which the activity in the original crude homogenate was far greater than that of the other enzymes (18.9×10^6 units; see legend to Table 1). Polyacrylamide-gel electrophoresis showed that the final preparation consisted of seven or eight protein bands (Plate 1), and gel-filtration studies (Fig. 1) revealed two main components of β-glucosidase with mol.wts. of about 70000 and 140000.

pH–activity profiles

β-Glucosidase activity was measured with the natural as well as with the synthetic substrate, and in both cases optimal enzyme activity was observed at pH4.5–5.0 (Fig. 2). There was a sharper peak of activity within this pH range when the natural substrate was used.

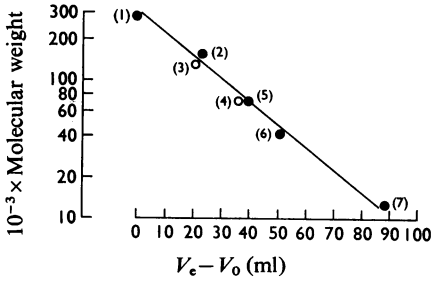


Fig. 1. *Estimation of the molecular weight of β-glucosidase by gel filtration*

Molecular weight of β-glucosidase and of reference proteins and Blue Dextran is plotted against the difference between elution volumes (V_e) for the reference proteins and void volume (V_0) (Andrews, 1965). (1) Blue Dextran; (2) γ-globulin; (3) β-glucosidase (high-molecular-weight); (4) β-glucosidase (low-molecular-weight); (5) bovine serum albumin; (6) peroxidase; (7) cytochrome *c*.

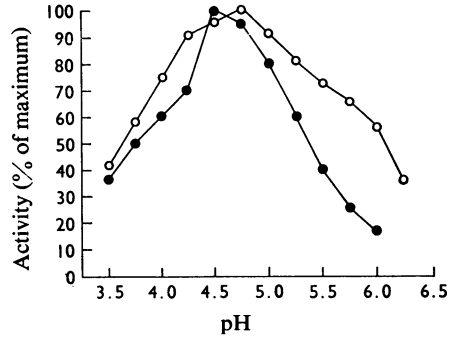


Fig. 2. *pH–activity profiles of β-glucosidase*

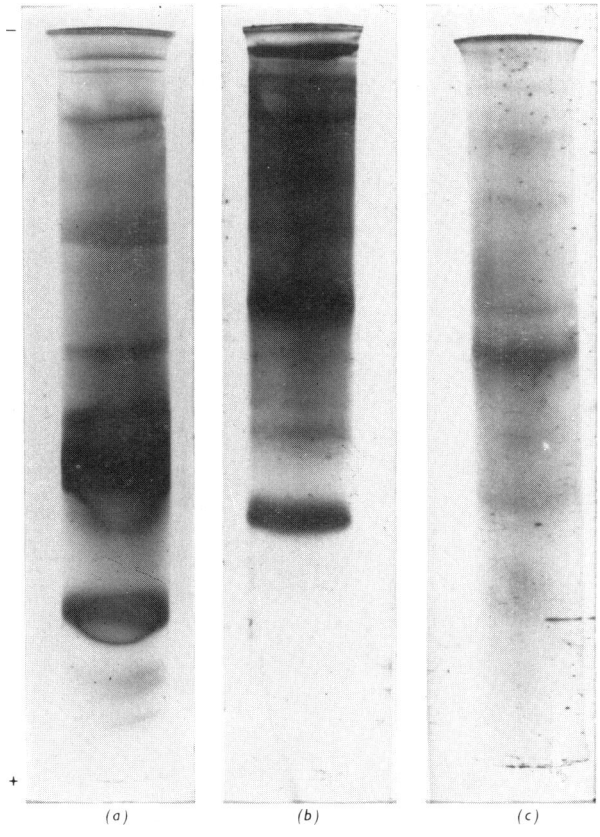
Assays of β-glucosidase activity with 4-methylumbelliferyl β-D-glucoside (○) or ^{14}C-labelled glucocerebroside (●; Ho *et al.*, 1973) were performed in the presence of 0.1% (w/v) Triton X-100 and 0.1% (w/v) sodium taurocholate (Ho, 1973).

Entrapment of β-glucosidase in liposomes

Table 2 shows the results from a typical study of entrapment of β-glucosidase and of other hydrolases in liposomes. Neutral liposomes composed of egg phosphatidylcholine and cholesterol consistently contained most (up to 100%) of the β-glucosidase used. Negatively charged phosphatidic acid liposomes entrapped a considerable proportion of the enzyme activity, and variation in the concentration of phosphatidic acid did not significantly alter entrapment values (65–85%, Table 2). It appeared that the inclusion of dicetyl phosphate and of the positively charged stearylamine diminished entrapment values to about 25 and 45% respectively. With all preparations, more than 95% of the liposome-associated enzyme activity was latent and could only be measured in the presence of Triton X-100 (legend to Table 2). Studies with neutral liposomes revealed that in addition to the entrapment of β-glucosidase there was concomitant capture of other hydrolases present in the preparation (Table 2). Although entrapment of three of the enzymes was modest (19–32%), that of α-galactosidase was practically total (99%).

Studies on liposome-entrapped β-glucosidase

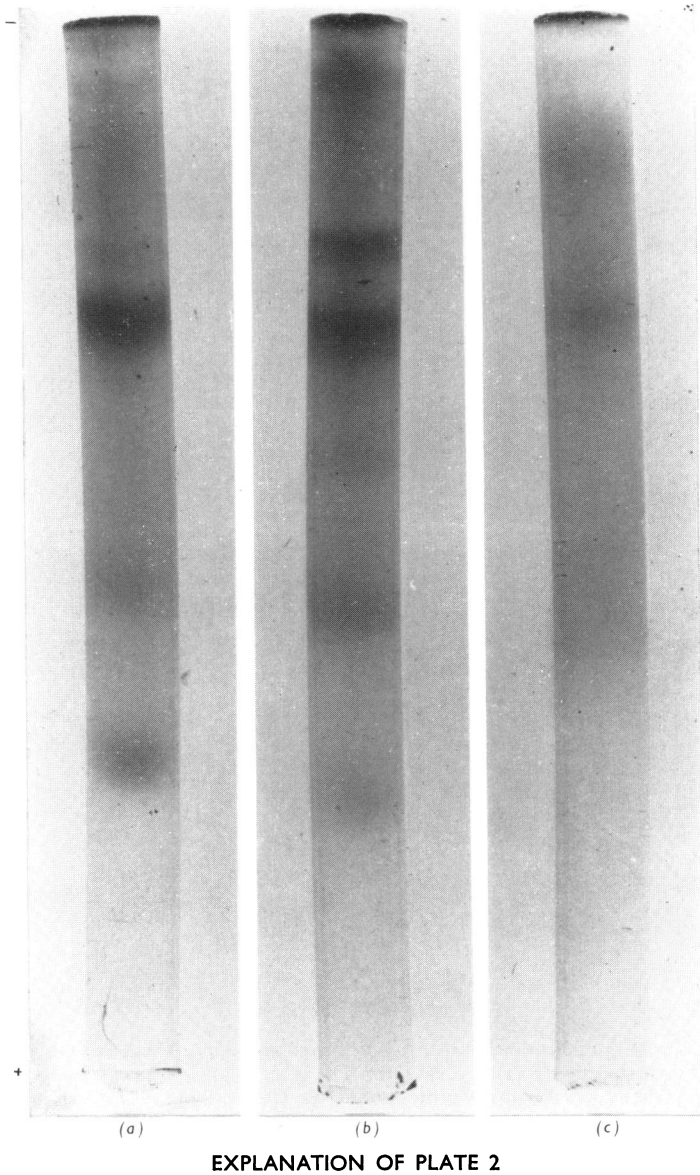
Extensive association of β-glucosidase (and of α-galactosidase) with neutral liposomes and the lesser degree of entrapment of other hydrolases (Table 2) suggested a specific interaction between liposomal lipids and certain enzymes in the enzyme preparation, discriminating against other protein components. This was confirmed in experiments in which the β-glucosidase-rich liposomal preparation before



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of samples from steps of purification of β-glucosidase

Gel electrophoresis was carried out (Davis, 1964) for 1 h. The following samples were applied to the gels: (*a*) 0.30 mg of protein from Step 2; (*b*) 0.11 mg of protein from step 3; (*c*) 0.03 mg of protein from step 4 (for explanation of steps see Table 1). Protein bands were detected with Amido Black.



EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of liposomal β-glucosidase: detection of protein

Samples of the liposomal preparation containing β-glucosidase before centrifugation (8.7 units, 0.07 mg of protein), unentrapped material in the supernatant (2.7 units, 0.09 mg of protein) and liposome-entrapped material in the suspended pellet (5.8 units, 0.02 mg of protein) after centrifugation were mixed with Triton X-100 (final concentration 0.8%) and applied to gels (*a*), (*b*) and (*c*) respectively. Before sampling, the supernatant was concentrated to one-tenth of its volume by dialysis against poly(ethylene glycol) 6000. Protein was detected with Coomassie Blue.

Table 2. *Entrapment of β-glucosidase and other hydrolases in liposomes*

Enzyme-rich material (2 ml), as obtained in the last step of the purification procedure (see Table 1) and containing approx. 8800 units of *N*-acetyl-β-glucosaminidase, 3600 units of α-mannosidase, 6300 units of α-glucosidase, 1100 units of α-galactosidase, 4500 units of β-glucosidase and 11 000 units of glucocerebroside β-glucosidase, was entrapped in liposomes composed of egg phosphatidylcholine (30.0 mg) and cholesterol alone or supplemented with charged lipids. Entrapped enzyme activity (% of activity used) was more than 95% latent and it could only be measured on the addition of Triton X-100 (0.8% final concn.).

Enzyme	Charged lipid	Molar ratio of lipids	Liposomal surface charge	Entrapment (%)
β-Glucosidase	None	7:2	Nil	100.0
	Phosphatidic acid	7:2:1	Negative	85.0
	Phosphatidic acid	7:2:2	Negative	65.0
	Phosphatidic acid	7:2:4	Negative	80.0
	Dicetyl phosphate	7:2:1	Negative	25.0
	Stearylamine	7:2:1	Positive	42.0
N-Acetyl-β-glucosaminidase	None	7:2	Nil	19.0
α-Mannosidase	None	7:2	Nil	32.0
α-Glucosidase	None	7:2	Nil	32.0
α-Galactosidase	None	7:2	Nil	99.0

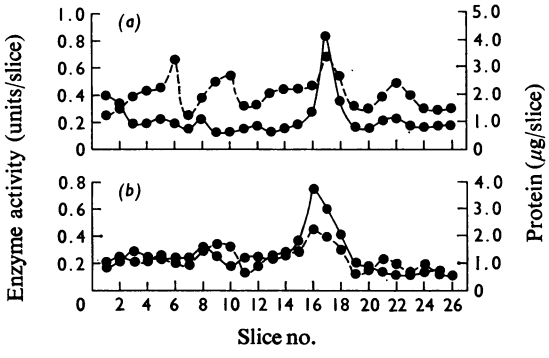


Fig. 3. *Polyacrylamide-gel electrophoresis of liposomal β-glucosidase: measurement of β-glucosidase and protein*

Samples of the β-glucosidase-rich liposome suspension before centrifugation (*a*, 11.2 units, specific activity 124.4 units per mg of protein) and the liposome-entrapped enzyme in the suspended pellet after centrifugation (*b*, 7.6 units, specific activity 230.3 units per mg of protein) were mixed with Triton X-100 (final concn. 0.8%) and subjected to electrophoresis on polyacrylamide gel. After electrophoresis, the gels were sliced and assayed for β-glucosidase activity (——) and protein (----).

centrifugation containing the entrapped and non-entrapped enzymes and the suspended pellet and supernatant after centrifugation were submitted to gel electrophoresis (Plate 2). Although six protein bands were observed in both the liposomal preparation before centrifugation and the supernatant containing free proteins, only three bands were visible in the liposome-entrapped material obtained by centrifugation. Further, the specific activity of the entrapped β-glucosidase had risen to twice the value obtained with the non-centrifuged preparation containing the unentrapped material as well (legend to Fig. 3). Measurement of β-glucosidase in the latter preparation after gel electrophoresis revealed a major peak of enzyme activity, which corresponded to the fifth of the six protein peaks detected (Fig. 3*a*). This peak was also the major one in the liposome-entrapped material (Fig. 3*b*).

Discussion

Application of liposomes as carriers of glucocerebroside β-glucosidase in the treatment of adult Gaucher's disease is hampered by the unavailability of the enzyme. Therefore our efforts have been focused on the development of a rapid procedure for obtaining a preparation rich in glucocerebroside β-glucosidase and in a form suitable for entrapment in liposomes and further clinical use. In a typical preparation (Table 1), the use of affinity chromatography (concanavalin A–Sepharose) followed by organic-solvent precipitation, enabled us to obtain from five placentas and in 2 days about 11 000 units of glucocerebroside β-glucosidase purified more than 1500-fold. In spite of the loss of approx. 95% of enzyme activity from step 3 to step 4 on addition of the ethanol/chloroform mixture, this step nevertheless eliminated the detergent used in the earlier step of tissue solubilization and much of the contaminating protein (Plate. 1), increased the specific activity of the enzyme fourfold (Table 1) and converted the enzyme into a form soluble in detergent-free buffer. Omission of the organic-solvent addition would have necessitated lengthy chromatography, imposing even

greater enzyme losses (Pentchev *et al.*, 1973). Experience in our laboratory has now shown that it is possible to prepare about 50000 units of the enzyme from 14–16 placentas in 8–10 days. Further, storage of individual enzyme preparations at −20°C has resulted in less than 10% loss of enzyme activity for preparations stored for 2 weeks and in less than 50% loss after storage for 2 months (I. P. Braidman & G. Gregoriadis, unpublished work). β-Glucosidase produced by the present method appeared to hydrolyse the natural as well as the synthetic substrate with optimal activity around pH4.5 (Fig. 2), which is typical for other acid hydrolases (Bouma, 1974).

In studies with liposomes, best entrapment values (nearly 100% of the enzyme used) for β-glucosidase were consistently obtained with neutral egg phosphatidylcholine–cholesterol liposomes, although the inclusion of phosphatidic acid only marginally decreased entrapment (Table 2). Considerably less enzyme activity was associated with negatively charged liposomes containing dicetyl phosphate or with positively charged liposomes containing stearylamine (Table 2). The substitution of the liposome-disrupting Triton X-100 with the much less toxic Triton WR1339 in the purification procedure (Table 1) decreased the likelihood of undesirable effects on the liposomal stability from traces of the detergent contaminating the final preparation. Indeed, enzyme-latency studies established that, for any of the liposome preparations, more than 95% of β-glucosidase was unavailable to the substrate (4-methylumbelliferyl β-glucoside) and was therefore presumed to be largely located within the outer boundaries of the liposomal structure. The quantitative association of β-glucosidase with liposomes, which is in contrast with the low entrapment values of other proteins (e.g. Gregoriadis & Ryman, 1972), suggests that only a small proportion, if any, of the entrapped enzyme is passively accommodated within the liposomal aqueous channels (water spaces between the lipid bilayers). Since glucocerebroside β-glucosidase is membrane-bound in its natural environment (Ho, 1973), it is more likely that most of the enzyme is associated with the liposomal lipids (e.g. egg phosphatidylcholine) through bonds the nature of which is unknown to us at present. In view of its discrimination against other proteins (Plate 2), and against some of the hydrolases, the entrapment of which was considerably lower (19–32%, Table 2), such bonding appears to be selective and to have contributed to further (twofold) purification of the enzyme (legend to Fig. 3). However, α-galactosidase was entrapped to an extent (99%) similar to that of β-glucosidase, and it appears that alterations in the liposomal lipid composition could increase entrapment values for a given hydrolase specifically. This should be of relevance in evaluating the use of liposomes in enzyme-replacement therapy of other lysosomal storage diseases.

The presence of other hydrolases in the liposomal preparation need not necessarily prejudice its use in man. For instance, it is conceivable that components (e.g. cofactors) contaminating the correcting enzyme in less-well-purified preparations may be essential for enzyme stability and full expression of activity *in situ*. Assuming that potentially detrimental action (e.g. allergic reactions or metabolic disturbances) of such components given with the enzyme via liposomes may be masked (Neerunjun & Gregoriadis, 1976; Gregoriadis *et al.*, 1974), the advantages gained from the present preparation of the glucocerebroside β-glucosidase-rich solution could outweigh possible drawbacks (e.g. immunological complications). These are often unavoidable even with highly purified enzymes of human origin (Eijsvoogel, 1974).

It is difficult at this stage to anticipate the quantities of glucocerebroside β-glucosidase and the frequency of administration needed for the elimination of substantial amounts of glucocerebroside deposited in the tissues of a given patient with Gaucher's disease and also to predict whether enzyme transported to the afflicted areas will enter the diseased lysosomes and exert its effect. Nonetheless, it has already been shown that in two patients who received 25000 and 55000 units (adjusted to nmol of substrate hydrolysed/min) respectively, the free enzyme, which like liposomes enters cells by endocytosis, is capable of degrading much of the stored substrate in the liver (Brady *et al.*, 1974). However, no improvement in the clinical condition of the patients was reported. It is hoped that, by using liposomes (as prepared in this study) which are known to leave the circulation rapidly and enter the tissues of the reticuloendothelial system (Gregoriadis, 1976*a*), loss of the enzyme to the periphery will be diminished. This, together with the expected (Gregoriadis & Buckland, 1973) intralysosomal release of glucocerebroside β-glucosidase, should render enzyme-replacement therapy of adult Gaucher's disease a rational, and perhaps more hopeful, exercise. This is indeed supported by preliminary results from a clinical trial conducted by this laboratory.

We thank Mrs. Daphne Bird for carrying out the gel electrophoresis.

References

Andrews, P. (1965) *Biochem. J.* **96**, 595–606

Bouma, J. M. W. (1974) in *Enzyme Therapy of Lysosomal Storage Diseases* (Tager, J. M., Hooghwinkel, G. J. M. & Daems, W. Th., eds.), pp. 197–206, North-Holland Publishing Co., Amsterdam and Oxford

Brady, R. O. & King, F. M. (1973) in *Lysosomes and Storage Diseases* (Hers, H. G. & Van Hoof, F., eds.), pp. 147–171, Academic Press, New York and London
Brady, R. O., Kanfer, J. N. & Shapiro, D. (1965) *J. Biol. Chem.* **240**, 39–43
Brady, R. O., Pentchev, P. G., Gal, A. E., Hibbert, S. R. & Dekaban, A. S. (1974) *N. Engl. J. Med.* **291**, 989–993
Braidman, I. P. & Gregoriadis, G. (1976) *Biochem. Soc. Trans.* **4**, 259–261
Colley, C. M. & Ryman, B. E. (1974) *Biochem. Soc. Trans.* **2**, 871–872
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
Eijsvoogel, V. P. (1974) in *Enzyme Therapy of Lysosomal Storage Diseases* (Tager, J. M., Hooghwinkel, G. J. M. & Daems, W. Th., eds.), pp. 125–130, North-Holland Publishing Co., Amsterdam and Oxford
Gregoriadis, G. (1976*a*) *N. Engl. J. Med.* **295**, 704–710
Gregoriadis, G. (1976*b*) *N. Engl. J. Med.* **295**, 765–770
Gregoriadis, G. (1976*c*) *Methods Enzymol.* **44**, 218–227
Gregoriadis, G. & Buckland, R. A. (1973) *Nature (London)* **244**, 170–172
Gregoriadis, G. & Neerunjun, E. D. (1974) *Eur. J. Biochem.* **47**, 179–185
Gregoriadis, G. & Neerunjun, E. D. (1975) *Biochem. Biophys. Res. Commun.* **65**, 537–544
Gregoriadis, G. & Ryman, B. E. (1972) *Eur. J. Biochem.* **24**, 485–491
Gregoriadis, G., Putman, D., Louis, L. & Neerunjun, E. D. (1974) *Biochem. J.* **140**, 323–330
Hers, H. G. (1973) in *Lysosomes and Storage Diseases* (Hers, H. G. & Van Hoof, F., eds.), pp. 147–171, Academic Press, New York and London
Ho, M. W. (1973) *Biochem. J.* **136**, 721–729
Ho, M. W., O'Brien, J. S., Radin, N. S. & Erikson, J. S. (1973) *Biochem. J.* **131**, 173–176
Juliano, R. L. & Stamp, D. (1976) *Nature (London)* **261**, 235–237
Kopaczyk, K. C. & Radin, N. S. (1965) *J. Lipid Res.* **6**, 140–145
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183–186
Neerunjun, E. D. & Gregoriadis, G. (1976) *Biochem. Soc. Trans.* **4**, 133–134
Patrick, A. D. (1965) *Biochem. J.* **97**, 17c–18c
Pentchev, P. G., Brady, R. O., Hibbert, S. R., Gal, A. E. & Shapiro, D. (1973) *J. Biol. Chem.* **248**, 5256–5261
Rahman, Y. E. & Wright, B. J. (1975) *J. Cell Biol.* **65**, 112–122
Robinson, D., Jordan, T. W. & Horsburgh, T. (1972) *J. Neurochem.* **19**, 1975–1985
Roerdink, F. H., Van Renswoude, A. J. B. M., Wielinga, B. Y., Kroon, A. M. & Scherphof, G. L. (1976) *J. Mol. Med.* **1**, 257–263
Segal, A. W., Wills, E. J., Richmond, J. E., Slavin, G. & Gregoriadis, G. (1974) *Br. J. Exp. Pathol.* **55**, 320–327
Weissmann, G., Bloomgarden, D., Kaplan, R., Hoffstein, S., Collins, T., Gotlieb, A. & Nagle, D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 88–92
Wisse, E., Gregoriadis, G. & Daems, W. Th. (1976) in *The Reticuloendothelial System in Health and Disease: Functions and Characteristics* (Reichard, S. M., Escobar, M. R. & Friedman, H., eds.), pp. 237–245, Plenum Publishing Corp., New York