Br. J. exp. Path. (1983) **64**, 670

TOXICITY OF SPHINGOMYELIN-CONTAINING LIPOSOMES AFTER CHRONIC INJECTION INTO MICE

E. A. H. WEERERATNE, G. GREGORIADIS AND J. CROW*

*From the Division of Clinical Sciences and *Section of Histopathology, Clinical Research Centre, Harrow, Middlesex HA1 3UJ*

Received for publication June 9, 1983

Summary.—Chronic treatment (10 i.p. injections over 20 days) of Balb/c mice with SM liposomes led to 50 and 300% enlargement of the liver and spleen respectively. No such effect was observed after similar treatment with PC liposomes. Biochemical analysis of the enlarged tissues showed no significant changes in the concentrations of glycolipid, phospholipid and certain hydrolytic enzymes. However, the increase in tissue size was paralleled by an increase in protein content. Light and electron microscopy studies of the enlarged tissues revealed an increase in the number of Kupffer cells and collections of inflammatory cells in the liver and widespread granulomatous inflammation in the spleen. We conclude that SM liposomes, although probably toxic for use as a drug carrier, may serve as a model agent in the study of tissue granulomatous inflammation.

AN IMPORTANT ASPECT of the use of liposomes as a drug carrier in therapy is their stability *in vivo*, namely the extent to which injected liposomes retain entrapped drugs en route to their destination (Gregoriadis, 1981). Recent work has shown that cholesterol (Kirby, Clarke and Gregoriadis, 1980) or sphingomyelin (Senior and Gregoriadis, 1982) in egg phosphatidylcholine liposomes reduces, through different mechanisms, the loss of the phosphatidylcholine component to plasma high density lipoproteins (HDL) and ensuing drug release through formed pores (Kirby and Gregoriadis, 1981) or following vesicle disintegration (Scherphof *et al.*, 1981). For instance, when liposomes are rich in cholesterol and sphingomyelin their stability is retained fully in the presence of plasma and also during their circulation in the blood of injected animals prior to uptake by the liver and spleen (Senior and Gregoriadis, 1982). In view of these findings, sphingomyelin has been used (*e.g.* Alving, 1982) for the preparation of stable liposomes capable of delivering drugs quantitatively to the reticuloendothelial system. However, in preliminary work (Weereratne *et al.*, 1982) with sphingomyelin liposomes chronically injected into mice we observed liver and spleen enlargement. In this report biochemical and morphological evidence is presented to show that such enlargement is associated with widespread granulomatous inflammation of the 2 tissues.

Address requests for reprints to G. Gregoriadis.

MATERIALS AND METHODS

Sources and grades of egg phosphatidylcholine (PC), bovine brain sphingomyelin (SM), cholesterol, 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferyl acetamidopyranoside have been described elsewhere (Senior and Gregoriadis, 1982; Braidman and Gregoriadis, 1977). Pure glucocerebroside was prepared from the spleen of a patient with Gaucher's disease type I by the method of Patrick (1965).

Preparation of liposomes.—Large oligolamellar liposomes were prepared (Szoka and Papahadjopoulos, 1978) from 33 μmol PC and equimolar cholesterol (PC liposomes) or from 33 μmol SM and equimolar cholesterol (SM liposomes). Solutions of the lipids in $CHCl_3$/methanol (2:1, v/v) were dried in 25 ml quickfit tubes by rotary evaporation of the solvents.

The lipids were then redissolved in 3 ml diethyl ether (PC liposomes) or 3 ml diethyl ether mixed with 3 ml chloroform (SM liposomes). To this solution 1·0 ml 0·01M sodium phosphate buffer containing 1% NaCl pH 7·4 (PBS) was added and the resultant two-phase system sonicated at 4° (PC) or 40° (SM liposomes) in a bath sonicator until a stable emulsion was formed, usually within 5 min. The final aqueous suspension of PC or SM liposomes was produced by removing the organic solvents and allowing it to stand for about 1 h at 4° or 40° respectively.

Detection and assay of lipids.—Total lipid was extracted (Folch *et al.*, 1957) from the mouse liver and spleen and stored in a known volume of chloroform under N_2 at −20°. Qualitative analysis of crude lipid extracts by thin layer chromatography (TLC) was accomplished using chloroform, methanol and water (65:25:4, v/v). The samples to be analysed and appropriate control lipids were applied onto precoated silica gel G plates activated overnight at 80° before use. Following chromatography, the lipids were visualized with I_2 vapour.

Glycolipids were separated from total tissue lipid extract by a slight modification of the method by Vance and Sweeley (1967). Total lipid extract was loaded onto silica acid columns (10 × 1 cm) equilibrated with chloroform, and neutral lipids were eluted with 50 ml of the solvent. Total glycosyl ceramides were then eluted with 100 ml acetone/methanol (9:1, v/v) with any phospholipids present being adsorbed onto the column. The glycosyl ceramide content of mouse liver and spleen was estimated by gas liquid chromatography using the purified lipid extracts from above (Purkiss, Hughes and Watts, 1978).

Protein.—A modification of the procedure of Lowry *et al.* (1951) enabling determination of membrane proteins was used.

Histology.—Liver and spleen from representative animals from control (untreated) and liposome-treated groups were used for morphological study. For standard histology the tissues were fixed in formalin and embedded in paraffin wax. Sections were stained with haematoxylin and eosin, periodic acid–Schiff (PAS) and PAS/diastase. For histochemistry frozen sections were cut. Acid phosphate and nonspecific esterase were used as lysosomal enzyme markers for cells of the macrophage cells series.

Electron microscopy (EM).—The tissue was fixed in 3% glutaraldehyde followed by 1% osmium tetroxide. Block staining with uranyl acetate was followed by dehydration in graded acetone solutions and embedding in Spurr resin. 1 μm sections were stained with toluidine blue. Thin sections were collected on copper grids, stained with Reynold's lead citrate and carbon coated before being viewed in a Philips 300 electron microscope.

Animal experiments.—Balb/C mice were injected i.p. with 0·3–0·35 ml PC or SM liposomes (2 mg phospholipid) once or 10 times at 48 h time intervals. Control animals received PBS by the same route as appropriate. Mice were killed 1, 5 and 10 days after the last injection. Liver and spleen were weighed and then processed for biochemical and morphological observations.

RESULTS AND DISCUSSION

Following a single injection of mice with PC or SM liposomes or 10 injections with PC liposomes no change in liver and spleen weights was observed. However, 10 injections of SM liposomes led to major spleen enlargement (Fig. 6) and to a less striking but significant hepatic enlargement (legend to Fig. 6). Whereas the average weight of a mouse spleen was 0·15 g, its average weight in the treated animals increased by nearly 300% to 0·44 g. By 5 days after the last injection, a 200% increase in spleen size was still present but this decreased to normal levels by Day 10. Average liver size was increased by about 50% and was reduced to normal by Day 5 (legend to Fig. 6). Since a total of only 20 mg sphingomyelin was injected in each of the animals, this could only account for a minor (6·6%) fraction of the increase in spleen weight, even assuming 100% uptake of the 10 doses of SM liposomes by the tissue and complete absence of catabolism of the lipid.

A variety of reasons for such tissue enlargement were considered. It is known, for instance, that senescent, pathological or chemically altered erythrocytes are selectively sequestered and degraded in the spleen. Part of the degradation process involves the catabolism of the several types of erythrocyte membrane lipids of which sphingomyelin is a main one. It has been shown by Kampine *et al.* (1967) that injection of erythrocyte stroma increases the rate of breakdown of erythrocytes (erythrocytorhexis). This results in liver and spleen enlargement and a concomitant increase in the specific activities of various hydrolytic enzymes in the 2 tissues,

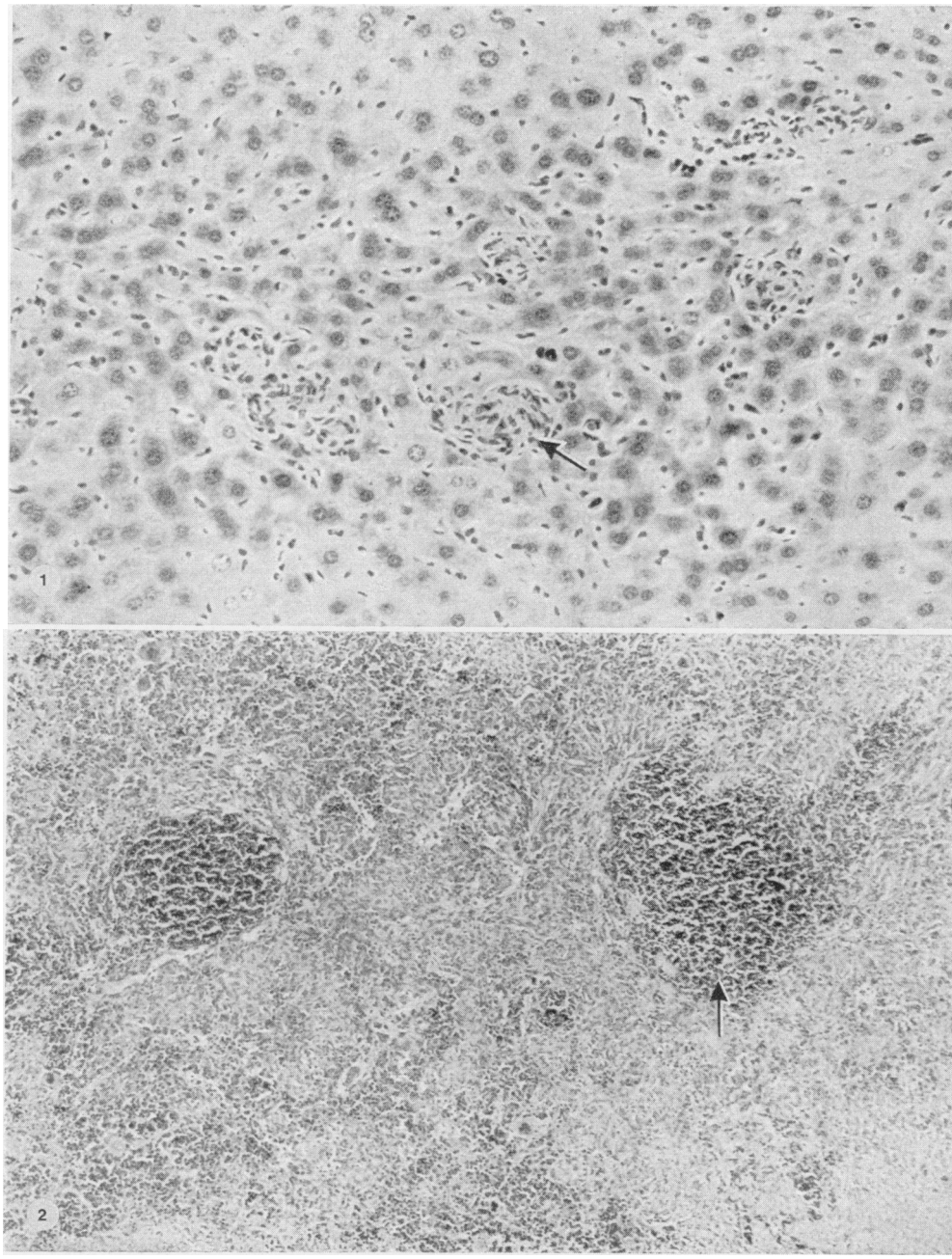
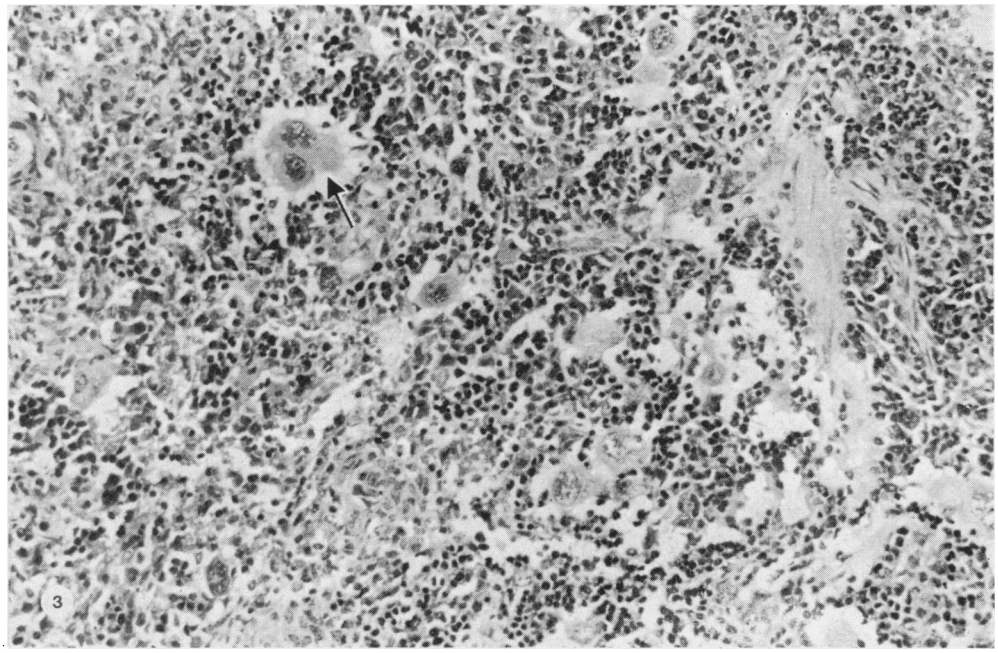


FIG. 1.—Liver section of a liposome-treated mouse showing proliferation of Kupffer cells and an infiltrate of inflammatory cells in the form of microgranulomas (arrow). Haematoxylin and eosin. × 50.

FIG. 2.—Spleen from a liposome-treated mouse showing diminution in the size of Malpighian corpuscles (arrows) surrounded by widespread granulomatous inflammation. × 20.



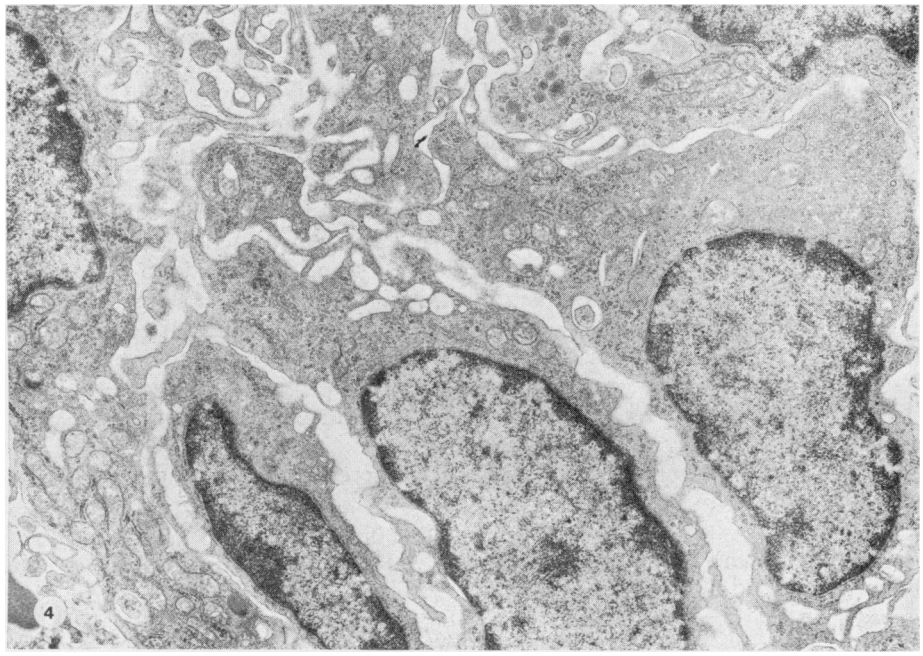


FIG. 3.—Spleen from a liposome-treated mouse showing increased number of megakaryocytes (arrow). × 50.

FIG. 4.—Spleen from a liposome-treated mouse showing epithelioid cells from an area of granulomatous inflammation with markedly interdigitating microbillous borders. × 8,316.

TABLE.—*Glucocerebroside: β-glucosidase in the tissues of mice treated with SM liposomes*

	Liver		Spleen	
	Treated	Control	Treated	Control
Total tissue activity (nmol min^{-1})	291·0±0·6	187·1±8·0	41·5±4·0	12·8±0·5
Total tissue protein (mg)	278·4±10·7	200·0±7·6	46·4±3·4	17·8±8·0
Specific activity (nmol min^{-1} mg^{-1})	0·83	0·90	0·85	0·75

Mice were injected i.p. 10 times with SM liposomes (Treated) or PBS (Control) at 48 h time intervals and killed 1 day after the last injection. Values for glucocerebroside: β-glucosidase and protein are mean±s.e. of 3 animals in each group.

especially the splenic glucocerebrosidase and sphingomyelinase. Indeed, injection of partially purified stromal sphingolipids consisting mainly of globoside and sphingomyelin causes a considerable change in the liver enzymes (Kampine *et al.*, 1967). It is, therefore, possible that in the present work the SM component of liposomes induced erythrocytorhexis, resulting in the loading of the liver and spleen with erythrocyte lipids and thus tissue enlargement.

Crude total lipid extracts from the livers and spleens of PBS and liposome-treated mice were compared for levels of glycosyl ceramide by glc and of sphingomyelin and phosphatidylcholine by TLC: no differences in the 2 groups were observed (not shown). The possibility of erythrocytorhexis leading to an increase in the levels of hydrolytic enzymes in the tissues capable of degrading erythrocyte glycopilids was also examined. Since glucocerebroside: β-glycosidase would belong to this class of hydrolases, its specific activity in the homogenates of the tissues of control and treated animals was assayed (Braidman and Gregoriadis, 1977). Again, there were no differences in the 2 groups (Table) and similar results were obtained with 2 other enzymes, β-galactosidase and β glucosaminidase (not shown). However, increase in tissue weight could be accounted for, at least in part, by an increase in total tissue protein. For instance, spleen and liver total protein content in the treated animals was 2·6 and 1·4 times greater respectively that in the controls (Table).

MORPHOLOGICAL OBSERVATIONS

As the results from biochemical observations of the liver and spleen of control and liposome-treated mice did not reveal any obvious differences, light and electron microscopic studies of the 2 tissues were undertaken. Fig. 1 from the liver of one of the liposome-treated animals shows an increase in the number of Kupffer cells and several small collections of inflammatory cells (microgranulomas). Confirmation that cells of the macrophage series were involved was obtained by positive staining with acid phosphatase and non-specific esterase reactions. Electron microscopy confirmed the presence of both lymphoid and macrophage cell types. Macrophages were noted to contain electron dense cell debris and an occasional small membranous structure was seen surrounded by macrophage cell processes.

On comparing the sections of spleens from treated and untreated animals, the most prominent feature in the treated mice was the expansion of the tissue between the Malpighian corpuscles (Fig. 2) as a result of the presence of widespread granulomatous inflammation. A marked increase in the number of megakaryocytes was also present (Fig. 3). The reason for the increased number of megakaryocytes in the spleen is difficult to explain. In the mouse, extramedullary haemapoiesis is a normal function of the spleen and it is possible that the injection of liposomes caused an intravascular coagulation syndrome with platelet consumption, hence the increased production of megakaryocytes as a compensatory process to replenish platelets. The extensive granulomatous inflammation seen by light microscopy was also evident by EM in the spleen of liposome-treated mice. Several granulo-

matous areas containing epithelioid cells were identified (Fig. 4). In addition, numerous active macrophages containing phagocytosed electron-dense material were observed (Fig. 5).

In conclusion, the most salient feature in the liver and spleen of mice treated chronically with SM liposomes was the presence of a diffuse inflammatory and granulomatous reaction. The enlargement of the 2 tissues was essentially due to an increase in their protein content. This did not appear to result from a net increase in a number of enzymes tested. It could be explained, however, as a result of the inflammation process observed: both an increased permeability of the tissue capillary walls to plasma proteins and infiltration of the tissue by inflammatory cells would presumably result in an increase in protein content. Since the 2 liposomal lipids, sphingomyelin and cholesterol are normally digestible by tissues of the reticuloendothelial system (Beaumier and Hwang, 1983; Poulos *et al.*, 1983) and are not known to be toxic, the granulomatous inflammatory response seen here is probably a reaction to the overloading of the system by the repeated injection of liposomes. Thus, it appears from the present study that large liposomes composed of sphingomyelin may be toxic for chronic *in vivo* use in man. This is unfortunate since sphingomyelin, in conjunction with cholesterol, greatly improves vesicle stability in the presence of blood and at the same time contributes to a

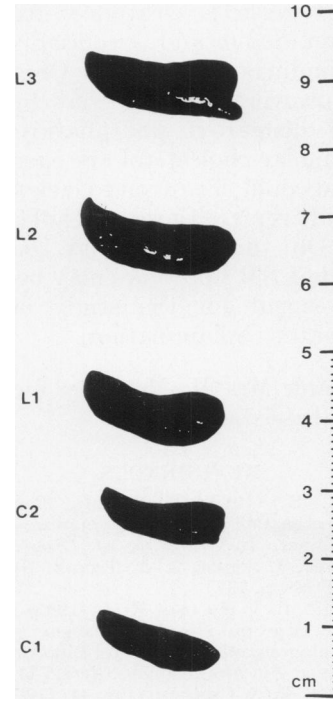


FIG. 6.—Typical spleens from control and liposome-treated mice 1 day after the last of 10 injections of PBS (C_1, C_2) and SM liposomes (L_1–L_3). Spleen weight (g) of treated mice (mean ± s.e., 12 animals) was 0·44 ± 0·04, 0·29 ± 0·08 and 0·16 ± 0·01 1, 5 and 10 days respectively after the last injection. Liver weight (g) of treated mice (mean ± s.e., 3 animals) was 2·06 ± 0·11, and 1·40 ± 0·08 1 and 5 days respectively after the last injection. The weight (g) of the tissues of control mice (mean ± s.e., 10 animals) did not change significantly in the course of the experiment and was 0·15 ± 0 (spleen) and 1·34 ± 0·03 (liver).

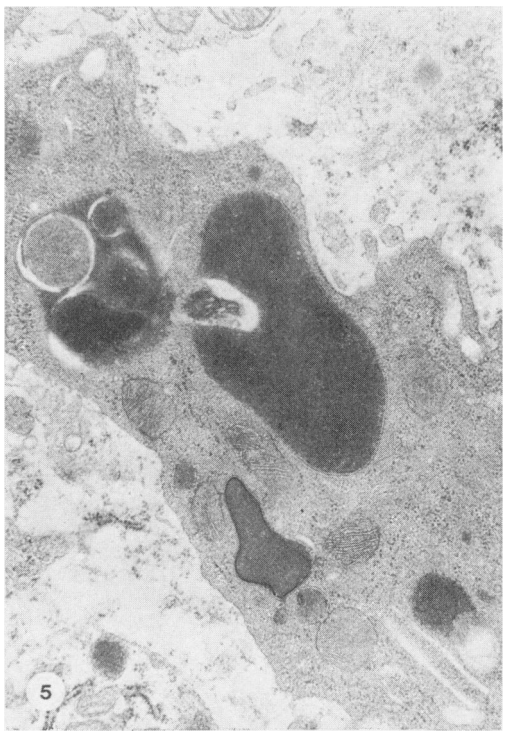


FIG. 5.—Spleen from a liposome-treated mouse showing electron dense phagocytosed debris. × 18,570.

prolonged vesicle clearance from the circulation (Senior and Gregoriadis, 1982). Recent findings (Senior and Gregoriadis, 1982), however, indicate that liposomes made of distearoyl phosphatidylcholine and equimolar cholesterol are exceedingly stable and could, if proven to lack toxicity, successfully replace those made of sphingomyelin. On the other hand, our data suggest that SM liposomes may be used as a model agent for the study of tissue granulomatous inflammation.

We thank Mrs M. Moriarty for expert secretarial assistance.

REFERENCES

ALVING, C. R. (1982) Therapeutic Potential of Liposomes as Carriers in Leishmaniasis, Malaria and Vaccines. In *Targeting of Drugs*. Eds G. Gregoriadis, J. Senior, & A. Trouet, New York: Plenum Press, p. 337.

BEAUMIER, P. L. & HWANG, K. J. (1983) Effects of Liposome Size on the Degradation of Bovine Brain Sphingomyelin/Cholesterol Liposomes in the Mouse Liver. *Biochim. Biophys. Acta*, **731**, 23.

BRAIDMAN, I. P. & GREGORIADIS, G. (1977) Rapid Partial Purification of Placental Glucocerebroside: β-glucosidase and its Entrapment in Liposomes. *Biochem. J.* **164**, 439.

FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. *J. Biol. Chem.* **226**, 497.

GREGORIADIS, G. (1981) Targeting of Drugs: Implicating in Medicine. *Lancet*, ii, 241.

KAMPINE, J. P., KANFER, J. N., GAL, A. E., BRADLEY, R. M. & BRADY, R. O. (1967) Response of sphingolipid hydrolases in spleen and liver to increased erythrocytorhexis. *Biochim. Biophys. Acta*, **137**, 135.

KIRBY, C. & GREGORIADIS, G. (1981) Plasma-induced Release of Solutes from Small Unilamellar Liposomes is Associated with Pore Formation in the Bilayers. *Biochem. J.*, **199**, 251.

KIRBY, C., CLARKE, J. & GREGORIADIS, G. (1980) Effect of the Cholesterol Content of Small Unilamellar Liposomes on their Stability *In vivo* and *In vitro*. *Biochem. J.* **186**, 591.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951) Protein Measurement with the Folin Phemol Reagent. *J. Biol. Chem.*, **193**, 265.

PATRICK, A. D. (1965) A Deficiency of Glucocerebrosidase in Gaucher Disease. *Biochem. J.* **97**, 17C.

POULOS, A., SHANKARAN, P., JONES, C. S. & CALLAHAN, J. W. (1983) Enzymatic Hydrolysis of Sphingomyelin Liposomes by Normal Tissues and Tissues from Patients with Niemann-Pick Disease. *Biochim. Biophys. Acta*. **751**, 428.

PURKISS, P., HUGHES, R. C., & WATTS, R. W. E. (1978) Screening of Urinary Oligosaccharides. A Quantitative Process. *Clin. Chem.* **24**, 669.

SCHERPHOF, G., DAMEN, J., HOEKSTRA, D., VAN RENSWOODE, A. J. B. M. & ROERDINK, F. H. (1981) Fundamental Studies on the Cellular Uptake of Liposomes. In *Cell Biological Aspects of Disease*. Eds Th.W. Daems, E. H. Burger & B. A. Afzelius. The Hague: Leiden University Press. p. 281.

SENIOR, J. & GREGORIADIS, G. (1982) Is Half-life of Circulating Small Unilamellar Liposomes Determined by Changes in Their Permeability? *FEBS Lett.*, **145**, 109.

SZOKA, F. & PAPAHADJOPOULOS, D. (1978) Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse Phase Evaporation. *Proc. Natl Acad. Sci. USA*, **75**, 4194.

VANCE, D. E. & SWEELEY, C. C. (1967) Quantitative Determination of the Neutral Glycosylceramides in Human Blood. *J. Lip. Res.* **8**, 621.

WEERERATNE, H., GREGORIADIS, G., BLAIR, H. & PURKISS, P. (1982) Liposomes and Gaucher Disease Type I. In *Advances in the Treatment of Inborn Errors of Metabolism*. Eds M. d'A. Crawfurd, D. A. Gibbs & R. W. E. Watts. Chichester: J. Wiley. p. 143.