review

The introduction of enzymes into cells by means of liposomes

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Defined, human genetic diseases due to the absence or malfunction of cytoplasmic enzymes can be divided into two main clusters. In the first, absence of appropriate hydrolytic enzyme activity ordinarily found within the vacuolar apparatus leads to the accumulation of uncleaved substrate in the lysosomes of affected tissue. Such diseases include Tay-Sachs disease, Gaucher's disease, peroxidase deficiency, and various mucopolysaccharidoses. Among the second group, in which enzyme activity is missing not from the lysosomes, but from non-membrane-bounded portions of the cytosol, are adenosine deaminase deficiency and the Lesch-Nyhan syndrome.

Since lysosomal storage diseases are caused by a genetic deficiency of specific acid hydrolases in lysosomes, reversal of such enzyme deficiencies has been approached by means of direct enzyme replacement. Because it is the affected system, the lysosomal apparatus of cells, that normally takes up extracellular macromolecules and particles by endocytosis, attempts have been made to mobilize the stored GM_2-ganglioside present in Tay-Sachs disease by direct administration of purified enzyme (1). Unfortunately, the injected enzyme disappears rapidly from the circulation and most of it becomes localized in the liver rather than at other affected sites, such as the central nervous system. Similar results are obtained when glucocerebrosidase and ceramide-trihexosidase are infused (2, 3). The problems associated with the direct administration of enzyme include *1*) the inability of this method to direct the enzyme to the parenchymal tissues containing the stored material, *2*) the potential antigenicity of the enzyme, *3*) the introduction of enzymes directly into the circulation where they may preemptively interact with their substrates or other proteins, and *4*) inability of free enzyme to cross the blood–brain barrier. The genetic disorders of lysosomes and attempts to correct them by using enzyme replacement therapy have recently been reviewed (4, 5).

To protect the enzyme against biodegradation upon infusion and to minimize the risk of a systemic immunological reaction, enzymes have been entrapped by various techniques prior to infusion. Catalase entrapped in synthetic, collodion–membrane microcapsules has been used for enzyme replacement in catalase-deficient mice (6, 7); microencapsulated L-asparaginase, injected intraperitoneally, has also been used successfully to suppress an implanted L-asparagine-dependent lymphosarcoma in C3HHeJ mice (7). The use of synthetic microcapsules in enzyme therapy is limited by their inability to be targeted to intracellular sites. A proposal for the usage of enzymes (asparaginase) entrapped in polyacrylamide gel particles of sizes suitable for either intravenous or intraperitoneal injection (less the 6 μm) (8) suffers from the inability of recipient animals to degrade the polyacrylamide matrix, thereby causing a long-term accumulation of the gel by cells of the reticuloendothelial system. The successful entrapment of β-glucosidase and β-galactosidase within erythrocyte ghosts has led to the proposal that enzyme-loaded erythrocytes be used in the treatment of lysosomal storage diseases, since such altered erythrocytes would probably be phagocytized by cells of the reticuloendothelial system (9).

A most promising development in the field of enzyme replacement therapy has been the use of artificial lipid vesicles for the entrapment of enzymes and for their delivery to cells, both in vitro and in vivo. The formation of such artificial lipid structures was first described in 1965 by Bangham and coworkers (10). By 1968 (11) we had coined the term

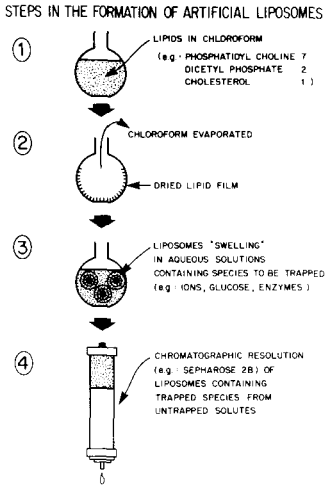

Fig. 1. Preparation of multilamellar liposomes.

"liposome" to describe these structures which, in their response to steroids, lytic proteins, and antibiotics, closely resembled natural biomembranes. Yet it was not until 1970 (12) that it became possible to encapsulate an enzyme (lysozyme), rather than low molecular weight molecules such as ions, glucose, or dyes, in the aqueous interstices between the multilamellar lipid bilayers of liposomes.

The aim of this review is to retrace the foundations of the use of liposomes as a potent tool in the entrapment of enzymes and in their delivery to cells, as well as to suggest directions for future investigations.

ENZYME ENTRAPMENT WITHIN LIPOSOMES

In our laboratory, liposomes are prepared in the following fashion (10, 13–16) (**Fig. 1**). Purified lipids are dissolved in chloroform and dried in vacuo, leaving a lipid film on the wall of the flask. Next, an aqueous solution of enzyme and/or other solutes that we wish to entrap is added. After brief mechanical shaking (Vortex for 10 min), the lipid dispersion is allowed to equilibrate for 2 hr. The resulting liposomes have been characterized as consisting of a series of concentric bilayers that alternate with aqueous compartments and are heterogeneous in size, ranging from 0.05–1.00 μm in diameter (17, 18). If unilamellar liposomes are required, the dispersion is briefly treated (20 min) with ultrasonic vibration in a bath-type sonicator; the resulting unilamellar liposomes are more homogeneous in size (0.05–0.10 μm diameter) (17, 18). For work with enzymes, multilamellar liposomes (**Fig. 2**) are preferred rather than unilamellar liposomes because of their greater entrapment capacity [hand-shaken multilamellar liposomes contain 1.8 ± 0.6 μl/μmol lipid whereas sonicated unilamellar liposomes contain 0.8 ± 0.3 μl/μmol lipid (19)] and because labile enzymes may be denatured by even brief sonication.

Deamer and Bangham (19) have recently described a preparative procedure for large unilamellar liposomes based on the "solvent evaporation" method of Papahadjopoulos and Watkins (18); liposomes formed when ether solutions of lipids are injected into warm aqueous solutions trap 14 ± 6 μl/μmol lipid. Experiments are currently in progress in our laboratory to show entrapment of biologically active proteins and enzymes by this method. The 10-fold improvement in entrapment capacity of these large unilamellar liposomes, as compared to multilamellar liposomes, may prove to be quite valuable for delivery of liposome-entrapped enzymes to cells.

The entrapment of any enzyme in the aqueous spaces between the lipid lamellae of liposomes must be demonstrated by the following evidence: *1*) resolution of the liposome-associated enzyme from the free enzyme by exclusion chromatography, *2*) latency of the liposome-entrapped enzyme, and *3*) correlation of increments in trapping with increments in lipid surface charge.

After formation of liposomes in their aqueous milieu, they are resolved from the untrapped enzyme and other solutes by gel filtration, using either Sephadex G200 or Sepharose 2B (Pharmacia, Piscataway, N.J.). The liposomes, being too large to enter the gel, typically elute immediately after the void volume, while the untrapped enzyme and solutes are retained by the gel and elute later in the bed volume. The quantity of enzyme associated with the lipid peak may be determined. In our experience, 6.9% of hexosaminidase A (15), 15.1% of lysozyme (12), and 5.8% of horseradish peroxidase (14) became entrapped by anionic liposomes. Moreover, the co-entrapment of glucose and/or CrO_4^{-2} in the enzyme-laden liposomes further indicates the integrity of the aqueous compartments.

Latency of the trapped enzyme may be judged by the inaccessibility of the substrate to the liposome-associated enzyme, as long as the substrate is incapable of diffusing across the lipid bilayers. Trapped enzymes may express their catalytic activities only after the structural integrity of the liposomes is disrupted by Triton X-100 (a nonionic detergent), amphotericin B, or nystatin (12). In contrast, free enzyme, as well as liposome-associated enzyme bound either electrostatically or hydrophobically to the exterior of the outermost lipid lamellae, may be assayed in the absence of detergent or polyenes.

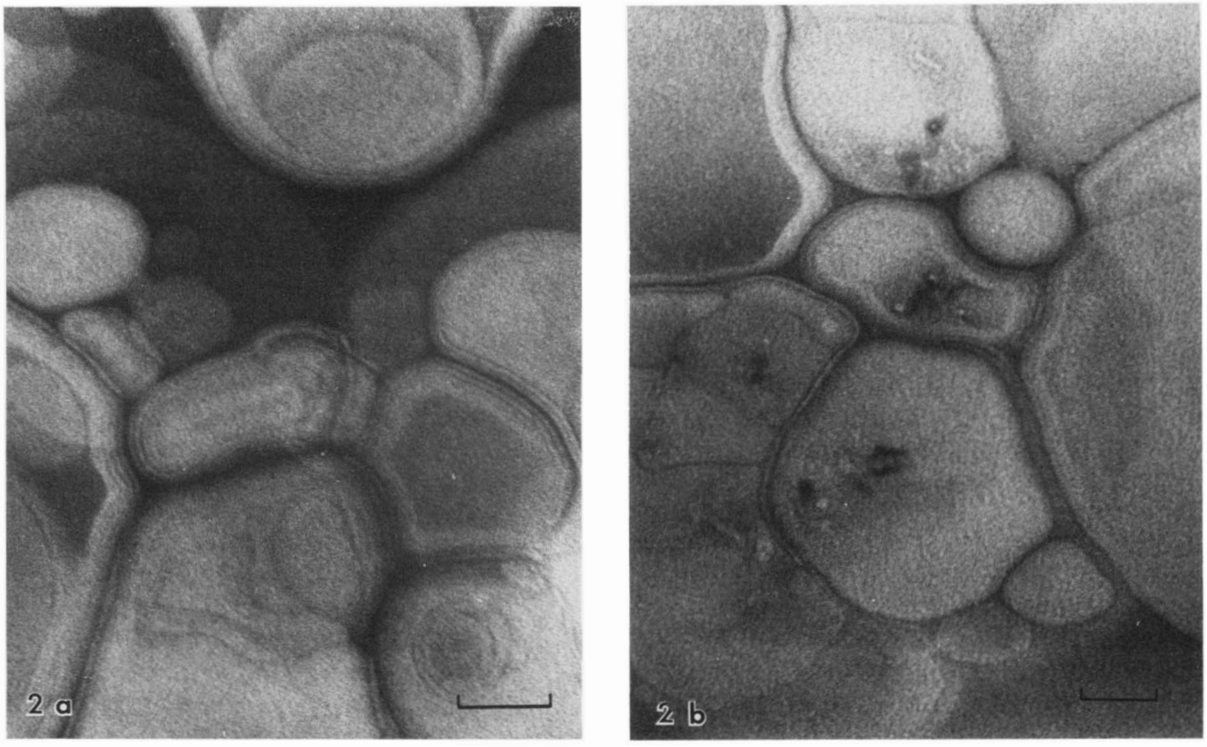

Fig. 2. (*a*) Multilamellar liposomes, L(PC 70:DCP 20:Chol 10), negatively stained with 2% ammonium molybdate in ammonium acetate buffer, pH 7.2. The multilamellar structure of these liposomes is evident as a result of packing during drying on the grid. Bar equals 60 nm. (*b*) Large unilamellar liposomes, L(PC 70:DCP 20:Chol 10), prepared by method of Deamer and Bangham (19) and negatively stained, as above. The majority of the liposomes are unilamellar. Bar equals 60 nm. Liposomes prepared courtesy of Mr. Hugh Schieren and micrographs courtesy of Dr. Sylvia Hoffstein.

Rechromatography of the pooled peak liposome fractions should give evidence of retention of the trapped enzyme and any other aqueous compartment markers (glucose, CrO_4^{-2}) within the lipid peak, whereas rechromatography of disrupted liposomes should show that the majority of the previously entrapped enzyme and aqueous compartment markers now elute independently of the lipid materials. This experiment excludes the possibility that the enzyme is nonspecifically adherent to, rather than enclosed within, the lipid bilayers.

The formation of liposomes depends upon the capacity of the phospholipids to swell and form hydrated liquid crystals that consist of a series of concentric bilayers that alternate with aqueous compartments. The volume of these compartments within which the enzymes may be entrapped is determined by the net charge of the lipids and by the ionic strength of the swelling solution (13, 17). We have observed that as the net anionic surface charge on the lamellae is increased by increasing the molar percentage of dicetyl phosphate from 5 to 10 to 20, capture of enzyme is also increased; like-sign repulsion of adjacent lipid layers increases the volume of the aqueous compartments. Evidence of increments in the entrapment within anionic liposomes of horseradish peroxidase (pI 7.2) and hexosaminidase A (pI 5.4), both of which are slightly anionic at pH 7.4 (swelling solution), and of the cationic enzyme lysozyme (pI 11.0) within cationic liposomes strongly suggests that these enzymes are free in the aqueous compartments; they are probably not entrapped as the result of nonspecific, electrostatic interactions (12, 14, 15).

Liposome nomenclature

In 1975 (14), we introduced a new, shorthand system of notation. All externally adherent materials are followed with a period, as in "aggIgG." (aggregated immunoglobulin G). Next is written "L" for liposomes; this is followed by the molar ratios of membrane lipids enclosed in parentheses, as in L(PC 7:DCP 2:Chol 1) to indicate phosphatidylcholine, dicetylphosphate, and cholesterol in the ratios indicated. All entrapped substances, such as enzymes or small molecules, are next written in square brackets, e.g., [hexosaminidase A, glucose] to designate liposomes after the capture of hexo-

TABLE 1. Enzymes incorporated into liposomes

Enzyme (source)	Lipid Composition	References
Lysozyme (egg white)	PC 7: DCP 2: Chol 1 PC 7: SA 2: Chol 1	12
Hexosaminidase A (human)	PC 7: DCP 2: Chol 1	15
Amyloglucosidase (*Aspergillus niger*)	PC 7: DCP 1: Chol 2	21, 22, 23
Peroxidase (horseradish)	PC 7: DCP 2: Chol 1 SPH 3: SA 1: Chol 1.1 PC 7: PA 1: Chol 2	14 24 25
β-D-Fructofuranosidase (yeast)	PC 7: PA 1: Chol 2	26, 27, 28
Dextranase	PC 7: PA 1: Chol 2	29, 30
α-Mannosidase (jack-bean)	PC 7: PA 1: Chol 2	31
Hexokinase (yeast)	PC 2: DCP 0.2: Chol 1.5 SPH 2: DCP 0.2: Chol 1.5	32 32
Glucose-6-phosphate dehydrogenase (yeast)	PC 2: DCP 0.2: Chol 1.5 SPH 2: DCP 0.2: Chol 1.5	32 32
β-Galactosidase (*E. coli*)	PC 2: DCP 0.2: Chol 1.5 SPH 2: DCP 0.2: Chol 1.5	32 32
Glucocerebrosidase (human)	PC 7: PA 1: Chol 2	33
β-Glucuronidase (bovine)	DPPC 7: SA 1: Chol 2 DPPC 7: PA 1: Chol 2	34 34
Neuraminidase (*Clostridium perfringens*)	PC 7: PA 1: Chol 2	35
Neuraminidase (influenza virus)	PC 9: DCP 1	36
Asparaginase (*Erwinia*)	PC 7: PA 1: Chol 2 PC 7: SA 1: Chol 2	37
Asparaginase (*E. coli*)	PC 10:DCP 1: Chol 2	38
Glucose oxidase (*Aspergillus niger*)	PI PC 7: PA 1: Chol 2 PC 7: SA 1: Chol 2	39 39 39
Cytochrome oxidase	PE 1: PC 1:CL 0.15:LL 0.25	40
Cytochrome *c* (horse heart)	PC 1: PE 1	17
Na^+, K^+-ATPase (*S. fecalis*)		20

Abbreviations used in table: phosphatidylcholine (PC), dicetylphosphate (DCP), cholesterol (Chol), stearylamine (SA), sphingomyelin (SPH), phosphatidic acid (PA), dipalmitoyl phosphatidylcholine (DPPC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), cardiolipin (CL), lysolecithin (LL).

saminidase A and glucose within the aqueous compartments. Proteins such as ATPase (20) actually incorporated in the lipid bilayers (**Table 1**) also belong between the parentheses, e.g., L(PC 7:DCP 2:Chol 1, ATPase).

LIPOSOME-MEDIATED ENZYME DELIVERY IN VITRO

Two mechanisms have generally been held responsible for the cellular uptake of liposomes (14, 15, 41–50). In the first, uni- or multilamellar liposomes may be endocytosed before entering the lysosomal apparatus (14, 15, 41–43). In the second, unilamellar liposomes may fuse their membranes with those of the target cell and introduce their contents directly into the cytosol (42, 44, 45); such liposomes are "leaky", and the loss of solutes into the surrounding milieu can be documented during the process of fusion (46). In addition, multilamellar liposomes might fuse with the plasmalemma of target cells, leading to the introjection of a liposome minus its outermost bilayer (41, 47, 50). The outermost aqueous compartment, which accounts for approximately 10% of the total trapped volume (11), will merge its contents immediately with the cytosol, while the remaining aqueous compartments re-

main lipid-bound. The intracytoplasmic liposome minus its outer bilayer may either be gradually degraded in situ or may undergo a secondary fusion event with membrane-bound organelles, such as the nucleus or the lysosomes.

The kinetics of liposome uptake via phagocytosis by mouse peritoneal macrophages was recently evaluated by Mattenberger-Kreber and coworkers (43). Saturable uptake of sonicated phosphatidylcholine liposomes radiolabeled with tracer amounts of either [^{14}C]- or [^{3}H]cholesterol was confirmed by increasing the concentration of liposomes. The time course of uptake was rapid with maximal uptake being attained within 30 min. Electron microscopy radioautography revealed that liposomes are sequestered within numerous peripheral cytoplasmic vacuoles within 10 min. Morphologically intact liposomes, as well as liposomal debris, is evident in vacuoles for up to 2 hr postincubation.

Uptake experiments (45, 48, 49) using cells that do not ordinarily engage in phagocytosis (Chinese hamster lung cells) have shown that both unilamellar and multilamellar liposomes exchange membrane lipids and trapped solutes with cells via vesicle–cell fusion and vesicle–cell lipid exchange. At 37°C, the vesicle–cell fusion interaction predominates whereas at 2°C, or when recipient cells are depleted of energy stores, vesicle–cell lipid exchange becomes significant. The fusion event is typified by the cellular association of both the aqueous compartment, containing [^{3}H]inulin, and the phospholipid component, labeled with [^{14}C]phosphatidylcholine, in exactly the same proportions as exist within liposomes. In contrast, under conditions favoring lipid exchange, the phospholipid marker became cell-associated while the aqueous compartment marker did not. Pagano and Huang (45) determined that the kinetics of uptake for unilamellar liposomes was independent of the liposome charge. Phagocytosis as a mode of uptake was ruled out on the basis of electron microscopic examination; radioautography confirmed the distribution of radioactive liposomal lipids throughout the cell rather than being localized in the lysosomal apparatus of the cell.

Using 3T3 mouse fibroblasts, Poste and Papahadjopoulos (42) observed uptake both by vesicle–cell fusion and by endocytosis. In a fluid state, negatively charged liposomes favor uptake by vesicle–cell fusion while neutral fluid liposomes and negative solid liposomes favor uptake via endocytosis. Uptake of solid vesicles is reduced by 80–90% by incubation with deoxyglucose (glycolysis inhibitor) plus sodium azide (respiration inhibitor) whereas, under similar conditions, uptake of "fluid" negative vesicles is reduced by only 30–40%. Pretreatment of cells with cytochalasin B, an inhibitor of phagocytosis, or prefixation with glutaraldehyde reduced the uptake of negative "fluid" vesicles by 20–32% as compared to 79–85% for "solid" vesicles. In addition, multilamellar liposomes behaved similarly to unilamellar liposomes.

In contrast to Poste and Papahadjopoulos' (42) claim that liposomes may enter fibroblasts either by fusion or by endocytosis, depending entirely upon the physical state (charge and fluidity) of the liposomal bilayers, Pagano and Huang (45) did not observe endocytosis of liposomes; furthermore, in their experiments, the magnitude of uptake by vesicle–cell fusion was not influenced by the charge of the liposomes.

In experiments performed with the phagocytic soil amoeba *Acanthamoeba castellani*, Batzri and Korn (41) have observed that the phospholipid composition of the liposome determines whether uptake by fusion or uptake by phagocytosis is to predominate. At 28°C, egg lecithin liposomes and dimyristoyl lecithin liposomes favor uptake via phagocytosis, while dipalmitoyl lecithin liposomes and distearoyl lecithin liposomes favor uptake via fusion. Phagocytic uptake was inhibitable either by 4°C incubation or by dinitrophenol. Positively charged egg lecithin liposomes were much more avidly taken up than neutral or negatively charged liposomes, but positively charged dipalmitoyl lecithin vesicles displayed only a slightly preferential stimulus for uptake as compared to neutral or negatively charged DPPC liposomes. The uptake by *Acanthamoeba castellani* of unilamellar DPPC liposomes, presumably by fusion, resulted in the loss of 60% of the aqueous contents (D-[^{3}H]glucose) of the vesicles (41). Grant and McConnell (46) similarly observed a 96% loss of entrapped solute upon fusion of unilamellar DPPC liposomes with the mycoplasma *Acholeplasma laidlowii*.

Experiments performed in our laboratory (50), in which cultured human lymphoid cells and human fibroblasts were incubated in the presence of multilamellar, anionic liposomes, L(PC 69:DCP 20:Chol 10:LL 1) [HRP] (horseradish peroxidase), showed that phagocytosis was not a significant mechanism of uptake. It was possible, for example, that some cells capable of phagocytosis may have contaminated the cell lines; however, noncytotoxic amounts of cytochalasin B, an inhibitor of phagocytosis, did not appreciably diminish uptake. The liposomes were essentially intact (94%) during incubation and entry of peroxidase into the cells could not be explained by its leakage from the liposomes and subsequent endocytosis by cells. As judged by ultrastructural

analysis, liposomes bearing enzyme did not simply adhere to the cultured cells, which is a problem when cationic liposomes encounter the anionic surfaces of natural membranes (24, 47). Finally, ultrastructural evidence was provided of the intracytoplasmic localization of liposomes still bearing cytochemically identifiable enzyme, a demonstration that has previously eluded other investigators (41, 42, 45). In two of the three cell lines studied, preincorporation of lysolecithin (a "fusogen") into the liposomal membranes was required in order to obtain significant levels of uptake; exogenous lysolecithin was ineffective.

A third type of interaction of liposomes with cells (in addition to endocytosis and fusion) is adsorption in which liposomes may attach to cell surfaces as a result of electrostatic attraction (24). Magee and coworkers (24) observed that cationic liposomes, L(SPH 3:SA 1:Chol 1.1), adsorbed to cultured HeLa cells with great avidity. Maximal uptake of entrapped horseradish peroxidase was observed within 30 min and exceeded by at least 300-fold that of free enzyme. Uptake was not inhibitable by incubation at 0°C, strongly suggesting adsorption. Electron microscopic examination of cells exposed to liposomes and subsequently stained in order to reveal peroxidase activity in situ showed *1*) the association of single liposomes, as well as of aggregates, with the cell surface in close proximity to pseudopodia, *2*) positive staining for peroxidase on sections of the outer surface of the cell membrane and pseudopodia, and *3*) membrane-bound cytoplasmic inclusions (phagocytic vacuoles) containing peroxidase-positive particulate, presumably liposomal, material. The authors (24) suggested that positive liposomes initially interact with negative cells via adsorption and are subsequently internalized by fusion and endocytosis.

TARGETING OF LIPOSOMES TO CELLS IN VITRO

Upon infusion into animals, liposomes have been shown to localize primarily in the liver and, to a lesser extent, in the spleen, regardless of the route of introduction (22, 26, 31, 34, 35, 60–62, 65, 67, 69–71). If liposomes are to be used successfully in the delivery of enzymes to the particular organ(s) or tissue(s) displaying a deficiency, techniques must be devised to bypass the accumulation of liposomes at unnecessary sites and to optimize the delivery to specific cell types. As we already have seen, the mode (fusion vs. endocytosis) and magnitude of cellular uptake of liposomes in vitro is determined by three physical parameters of the liposome: *1*) phospholipid composition (fluidity), *2*) charge, and *3*) size. Conceivably, these parameters may be altered to favor accumulation of liposomes by a particular cell type. The most potentially direct approach to targeting is offered by the use of surface macromolecular ligands. The following discussion reviews the efforts of three laboratories in this area of liposome engineering.

We have observed that liposomes coated with aggregated IgG provide a far better endocytic stimulus than uncoated liposomes. Aggregated, rather than native, immunoglobulins preferentially coat and partially insert into liposomes. Over 98% of the aggregated human IgG became associated with L(PC 70:DCP 20:Chol 10) by both electrostatic and hydrophobic association when AggIgG was presented at a concentration of 10 μg/μmol phospholipid (51). Since aggregated immunoglobulins form lattices in which the key Fc regions are disposed toward the interior of the outermost lamellae of liposomes and also toward the surrounding medium, it would be expected that these would act as ligands for the Fc receptors of the polymorphonuclear leukocytes (PMN) and consequently provoke endocytosis (52). In fact, hexosaminidase A was more actively taken up when presented to Tay-Sachs phagocytes as AggIgG.L [Hex A, glucose] than when presented in liposomes coated with native IgG or in uncoated liposomes (15) (**Fig. 3**). Similarly, the uptake of AggIgM.L [HRP, glucose] by dogfish phagocytes exceeded that of free enzyme by 120-fold, native IgM-coated liposomes by 60-fold, and uncoated liposomes by 50-fold (14). Immunoglobulin-coated liposomes presumably enter phagocytes as a result of an internalization of the cell membrane that contains the aggregated immunoglobulin–Fc receptor complex (15).

Gregoriadis and Neerunjun (52) have described a scheme for cell strain-specific homing of liposomes; the uptake of liposomes is enhanced several times (3- to 25-fold) when cells of a particular strain (HeLa cells, AKR-A mouse leukemia cells, human fibroblasts) encounter liposomes associated with IgG immunoglobulins (presumably coated with IgG) raised against the particular cell strain. The association of liposomes with cells during the incubation was monitored by use of the radiolabeled markers ^{125}I-labeled IgG, ^{111}In-labeled bleomycin, and cholesteryl [1-^{14}C]palmitate. The proportions of the cell-associated radiolabels remained quite similar to their proportions in the liposomes, suggesting the integrity of the liposomes. By subcellular fractionation, 79.5% of the entrapped bleomycin was recovered from the lysosome-rich particulate fraction and 20.5% was re-

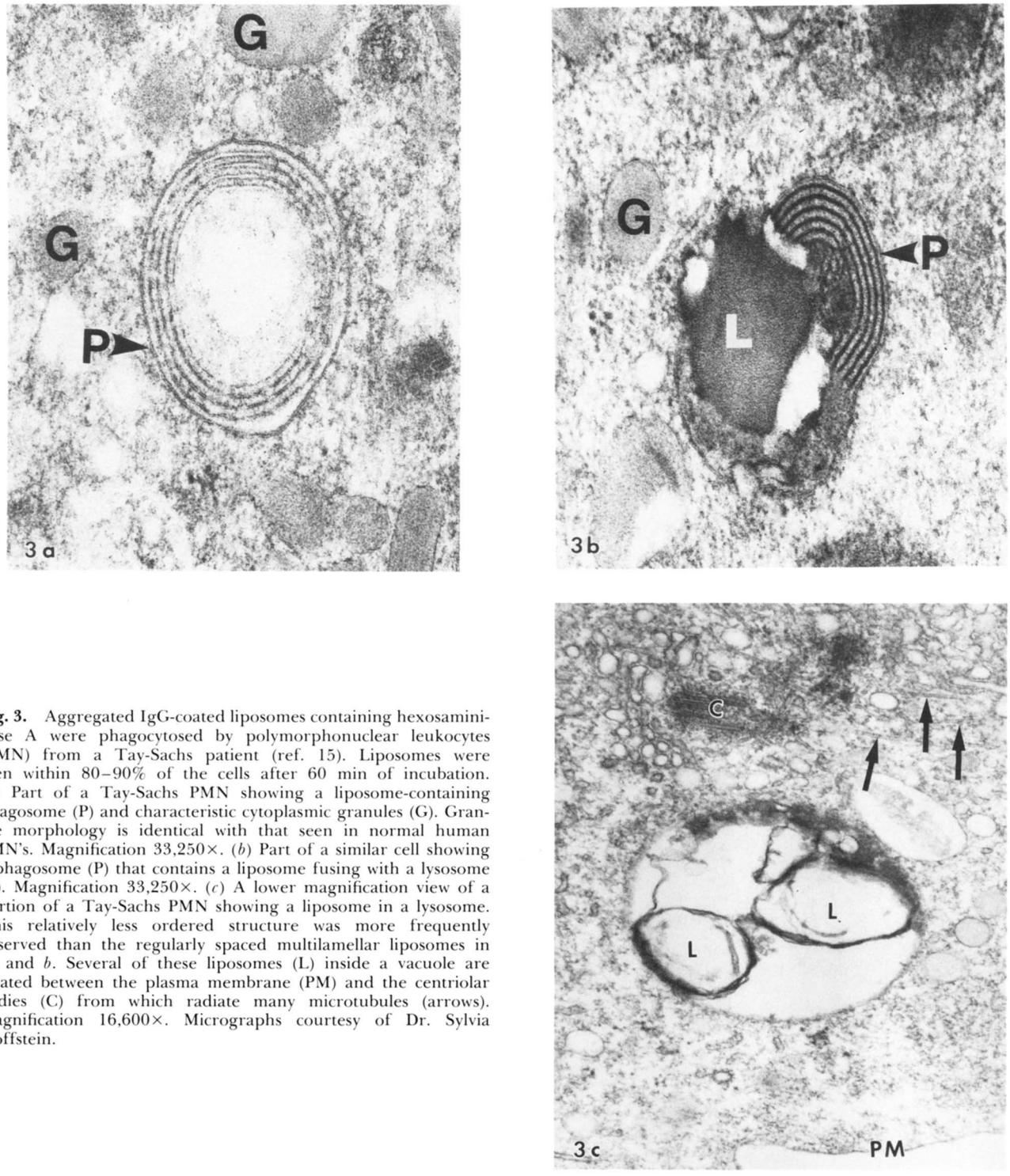

Fig. 3. Aggregated IgG-coated liposomes containing hexosaminidase A were phagocytosed by polymorphonuclear leukocytes (PMN) from a Tay-Sachs patient (ref. 15). Liposomes were seen within 80–90% of the cells after 60 min of incubation. (*a*) Part of a Tay-Sachs PMN showing a liposome-containing phagosome (P) and characteristic cytoplasmic granules (G). Granule morphology is identical with that seen in normal human PMN's. Magnification 33,250×. (*b*) Part of a similar cell showing a phagosome (P) that contains a liposome fusing with a lysosome (L). Magnification 33,250×. (*c*) A lower magnification view of a portion of a Tay-Sachs PMN showing a liposome in a lysosome. This relatively less ordered structure was more frequently observed than the regularly spaced multilamellar liposomes in 3*a* and *b*. Several of these liposomes (L) inside a vacuole are located between the plasma membrane (PM) and the centriolar bodies (C) from which radiate many microtubules (arrows). Magnification 16,600×. Micrographs courtesy of Dr. Sylvia Hoffstein.

covered from the membrane fraction. These authors' conclusion that the liposomes are internalized by cells via endocytosis could have been strengthened by the presentation of electron microscopic evidence for the in situ localization of liposomes to the lysosomal apparatus, as well as for control in vitro incubations in the presence of cytochalasin B, incubation at 0°C, or incubation in the presence of metabolic inhibitors (e.g., dinitrophenol, azide, or rotenone).

Juliano and Stamp (53) report the incorporation of the major sialoglycoprotein of human erythrocyte membranes into liposomal membranes; this glycoprotein displays activity as a receptor for the lectins wheat germ agglutinin (WGA) and phytohemagglutinin (PHA), but not for concanavalin A. The glycoprotein was considered likely to have been displayed on the outer surface of the liposome since it was released from the liposome by treatment with trypsin under conditions that failed to release [^{3}H]sucrose entrapped within the aqueous compartment. After 4 hr of incubation, sialoglycoprotein liposomes displayed five-times as much agglutination with erythrocytes in the presence of WGA as in the absence of lectin. Post-treatment of the WGA-induced cell–liposome aggregates with the haptene sugar *N*-acetylglucosamine, at concentrations capable of preventing the formation of the aggregates, failed to reverse the aggregation; this observation was taken to indicate cellular internalization of the liposomes. Electron microscopic evidence supporting this conclusion was not presented.

LIPOSOME-MEDIATED ENZYME DELIVERY IN VIVO

In vivo liposomal "latency"

The latency of an entrapped enzyme or marker is a function of the integrity of the liposomal membrane. Latency is experimentally defined as the incapacity of the entrapped enzyme to act on exogenous substrate unless the liposome is disrupted. This definition may be misleading when "latent" liposomes are infused into animals; the in vivo integrity of the liposomal membrane may be compromised by complement-mediated damage, by the action of phospholipases, or by the action of hydrophobic plasma proteins. The phospholipid composition of the liposome may determine its susceptibility to disruption while the conformation of the entrapped molecule and the nature of its entrapment determine whether or not it will remain lipid-associated. In vivo investigations of the fate of injected liposomes by using entrapped aqueous phase markers may lead to invalid conclusions unless the liposomes remain intact while in circulation. The ideal control experiment would be to rechromatograph liposomes recovered from the plasma minutes after injection. As a minimum, markers of the aqueous and lipid components should be shown to remain in the same proportions during their clearance from the plasma and their localization in tissues as exist in intact liposomes.

In their examination of the fate of mouse liposomes containing $^{22}Na^+$ and $^{86}Rb^+$ injected into mice, Kimelberg and coworkers (54) observed that the entrapped markers followed more closely the distribution of free markers than that of a lipid marker. Their suspicion that the entrapped cations leaked from the liposomes and were accumulated by the tissues independently of liposomes was confirmed by their finding that in vitro incubation of liposomes in 30% mouse serum causes release of 44% of the entrapped $^{22}Na^+$ within 2 hr. Similarly, Gregoriadis (55) observed that the time course of plasma clearance of liposome-entrapped penicillin followed more closely that of free penicillin than that of liposomes, and also suggested that the drug was lost by accelerated diffusion through the liposomal membranes. Arakawa and coworkers (56) showed that the rate of clearance of drugs from intramuscular sites after injections of drug-loaded liposomes was inversely proportional to the liposomal cholesterol content. In vitro leakage studies revealed that serum accelerated the leakage of [^{14}C]cefazolin but, by increasing the liposomal cholesterol content, the rate of leakage was reduced. Recently, Krupp, Chobanian, and Brecher (57) presented evidence that unilamellar liposomes containing egg lecithin and cholesteryl oleate change in density and size within 5 min of injection into rats or incubation in plasma; they suggest that segments of the liposomal membrane become associated with plasma high density lipoproteins.

The further possibility exists that liposomes activate the complement pathway in vivo and this poses yet another potential challenge to liposomal integrity. The ability of complement to mediate the lysis of liposomes has been well documented (32, 58, 59); the membrane lesions resulting from complement-mediated damage are large enough to permit loss of enzymes as large as β-galactosidase (mol wt 518,000) (32).

In vivo fate of liposomes

Kriss, Dunnick, McDougall and their colleagues (60–63) have performed a most comprehensive study of the in vivo distribution of liposomes as a function of their phospholipid composition, size, interaction with antibodies and proteins, and route of administration. Their findings bear on the targeting of enzyme-laden liposomes in vivo.

Liposomes (62), L(PC 11:gangliosides 1), that had been sonicated for 5 min (900 Å diameter) thus entrapping the radioactive ion pertechnetate, $^{99m}TcO_4^-$, and/or the spin label tempocholine chloride, were administered intravenously to mice (60). Within 30 min, 90% of the entrapped radioactivity had been

cleared from circulation and the examination of electron paramagnetic resonance (EPR) spectra for blood specimens indicated that liposomes remaining in circulation were, for the most part, intact. Autopsy of mice as soon as 5 min after injection revealed the accumulation of 25% of the injected radioactivity in liver, 2% in spleen, 2% in kidney, 2% in lung, and 2% in stomach. Accumulation of liposomes by the liver was doubled and accumulation in the stomach was reduced either by increasing the duration of sonication of the liposomes, L(PC 11:gangliosides 1), or by incorporating cholesterol into the liposomes, L(PC 11:gangliosides 1:Chol 8.2). The similarity of the in vivo effects of prolonged sonication of liposomes and addition of cholesterol to liposomes suggested to the authors that both manipulations caused the liposomes to be less "leaky". In fact, in vitro efflux studies (dialysis method) confirmed this interpretation. The authors further hypothesize that sonication in air causes oxidation of unsaturated fatty acids, consequently resulting in bilayers with a more rigid conformation; incorporation of cholesterol into lipid bilayers also decreases membrane fluidity and, in turn, permeability. The incorporation of charged phospholipids (phosphatidic acid, phosphatidylethanolamine, phosphatidylserine) into the standard liposomes did not significantly alter their in vivo distribution.

Next, the effect of the route of administration on the in vivo fate of liposomes, L(PC 11:gangliosides 1) [$^{99m}TcO_4^-$], that had been sonicated for 60 min (500 Å diameter) (62) was evaluated (61). The organ and tissue distribution was qualitatively similar following either intravenous, intraperitoneal, or subcutaneous injection. Quantitatively, however, use of the intraperitoneal route, as compared to the intravenous route, results in an 8-fold reduction in liver and spleen accumulation, and subcutaneous injection results in a 21-fold reduction in liver and a 50-fold reduction in spleen accumulation. Recovery in lung was reduced approximately 2-fold by intraperitoneal and subcutaneous injections. In addition, Dapergolas, Neerunjun, and Gregoriadis (39) reported that intragastric administration to rats of ^{125}I-polyvinylpyrrolidone-loaded liposomes results in the recovery of 98% of the marker in the feces; only 0.2% of the injected load was recovered in the liver, regardless of liposome charge. Furthermore, liposomes administered intramuscularly enter the circulation and display significant hepatic accumulation only if they have been sonicated for at least 20 min; unsonicated liposomes injected intraperitoneally are capable of passing through the vascular membranes and into circulation, although less rapidly than sonicated liposomes (39). These results imply that intraperitoneal and subcutaneous routes of administration may serve to bypass the rapid sequestration of liposomes in liver and spleen, thereby yielding a time-capsule effect—the systemic release of liposomes at slower rates over longer periods of time.

Finally, Dunnick et al. (62, 63), examined the ability of various macromolecules (polyamino acids, immunoglobulins, human thyrotropin, human antithyroglobulin) to act as surface ligands and to direct the in vivo distribution of unilamellar liposomes, L(PC 11:gangliosides 1) [$^{99m}TcO_4^-$]. The accumulation of liposomes in liver and spleen following intravenous infusion was increased 3-fold for poly-L-phenylalanine vesicles and 2-fold for poly-L-tyrosine vesicles (62). Liposomes coated with IgG also displayed a 2-fold increase in hepatic and splenic accumulation, as well as a 2- to 3-fold increase in lung accumulation, following intravenous injection (62). Liposomes containing gangliosides were shown to bind human antithyroglobulin and human thyrotropin 2–5 times more efficiently than liposomes lacking gangliosides (63). The binding of bovine thyrotropin also displays a requirement for gangliosides (64). The tissue distribution following intravenous administration of ^{125}I-antithyroglobulin IgG.L(DPPC 11:gangliosides 1:Chol 8.2) is similar to that for uncoated liposomes; cholesterol is required to stabilize the antibody–liposome interaction in vivo.

The in vivo fate of liposomes, L(PC 7:DCP 1:Chol 2), containing either ^{131}I-labeled albumin or [^{3}H]amyloglucosidase in their aqueous compartments and [^{3}H]cholesterol in their lipid bilayers was determined (22, 65). Approximately 70% of the liposomal markers are cleared from circulation within 15 min of intravenous injection and are concomitantly accumulated by the liver (56%) and spleen (12%); subcellular fractionation of liver reveals that approximately half of the enzyme marker is in the mitochondrial/lysosomal fraction. Gregoriadis and Ryman (22, 65) have determined that the ratio of [^{3}H]cholesterol (lipid phase marker) to ^{131}I-labeled albumin (aqueous compartment marker) is constant for liposomes circulating in the blood for as long as 250 min. Their conclusion that liposomes remain intact during circulation is valid for albumin-containing liposomes and may not be extrapolated to liposomes trapping other proteins and markers. In fact, the apparent latency described for L[^{131}I-labeled albumin] may reflect the inability of albumin to dissociate from damaged liposomes; albumin has been shown to engage in hydrophobic and electrostatic interactions with liposomal bilayers (66).

The time course of clearance from the plasma after an intravenous injection of neutral, L(PC 7:Chol 2), or negatively charged liposomes, L(PC 7:PA 1:Chol 2) and L(PC 7:DCP 1:Chol 2), displays a prominent rapid phase whereas positive liposomes, L(PC 7:SA 1:Chol 2), do not; the slow phase displays a similar rate regardless of liposome charge (28). Furthermore, the rate of hepatic accumulation is proportional to the rate of plasma clearance (28). The rapid phase could be reduced for negative liposomes (3.4 mg lipid) by simultaneously injecting a large dose of either negative or positive liposomes (13.2 mg lipid) and could be eliminated for positive liposomes by the same technique, suggesting that the rapid phase represents a common mechanism of tissue accumulation for liposomes regardless of charge (22, 28, 65). Juliano and Stamp (67) and others (68, 69) have confirmed the biphasic kinetics of liposome elimination from the plasma and the preferential clearance of negative liposomes as compared to positive ones. Caride and coworkers (70) have since shown that the hepatic accumulation of neutral liposomes, L(PC 3:Chol 3.8) [^{99m}Tc-*sn*-diethylene triamine pentaacetic acid], could be reduced significantly by injecting mice with unlabeled liposomes 30 min prior to injections with labeled liposomes; in contrast to Gregoriadis' results, they also observed increases in uptake by spleen and lung of 2-fold and 4-fold, respectively.

The charge of liposomes also influences their rate of accumulation by organs other than the liver. Splenic uptake of large negative liposomes containing either [^{14}C]EDTA (69) or [^{14}C]poly(I)·poly(C) (71) is approximately 2-fold greater than for positive liposomes. Uptake by brain and lung tissue displays a 2- to 4-fold preference for positive, as compared to negative, liposomes containing [^{14}C]EDTA (69). In contrast, Kimelberg (68), using *Macaca* monkeys as recipients for small sonicated liposomes containing [^{3}H]methotrexate, has observed a preference by brain, spleen, and bone marrow for positive liposomes and by lung for negative liposomes. Presumably size as well as charge contribute to determination of liposomal fate in vivo.

The size of liposomes has been shown to affect their kinetics of clearance from the bloodstream. Juliano and Stamp (67) and also Kimelberg (68) report that small unilamellar liposomes, produced by prolonged sonication, were cleared less rapidly (t½ 200 min) than large multilamellar liposomes (t½ 22 min) after intravenous infusion. Furthermore, small sonicated liposomes are recovered in the plasma at approximately 5-fold higher levels than unsonicated liposomes following intraperitoneal injection and at approximately 10-fold higher levels following intramuscular injection (39). These results suggest that large liposomes are limited in their ability to penetrate vascular membranes and to pass through small capillaries.

Next, Gregoriadis and Ryman (26) confirmed the in vivo distribution pattern of radioactive liposome markers (^{131}I-labeled albumin, [^{3}H]amyloglucosidase, [^{3}H]cholesterol) (22, 65) by using a high specific activity enzyme, β-fructofuranosidase (invertase), entrapped within liposomes L(PC 7:PA 1:Chol 2). Within one hour of intravenous infusion, half of the enzyme activity, said to be latent, was cleared from circulation. By 6 hr, 45% of the injected enzyme was in the liver and 10% was in the spleen; after 48 hr, 25% still remained in the liver and, after 100 hr, 5% remained. Subcellular fractionation of liver again revealed that nearly half of the intracellular invertase was present in the mitochondrial/lysosomal fraction. These early findings have since been confirmed with demonstrations of a similar fate of intravenously infused α-mannosidase-loaded liposomes (31) and neuraminidase-loaded liposomes (35); liposomes accumulate in the liver and spleen where they are localized intracellularly, mostly to the lysosome-rich fraction, and express their entrapped enzyme's catalytic activity in situ for several days.

Most recently, Steger and Desnick (34) examined the in vivo tissue and subcellular fate of β-glucuronidase entrapped in sonicated liposomes after intravenous infusion into mice. Enzyme entrapped in either negative, L(DPPC 7:PA 1:Chol 2), or positive, L(DPPC 7:SA 1:Chol 2), liposomes was rapidly cleared from circulation and accumulated in the liver. Approximately 50–80% of the injected entrapped enzyme was in the liver after 1 hr and was stable for 48 hr, thereafter diminishing gradually over 8–11 days. Renal accumulation and retention of enzyme appeared enhanced when the enzyme was packaged in negative, rather than positive, liposomes.

The fate of [^{3}H]cholesterol-labeled liposomes was strongly dependent upon the enzyme entrapped, presumably within (34). Enzyme-loaded liposomes display a more rapid hepatic accumulation, of a greater magnitude, and are retained in the liver for approximately 9 times as long as buffer-loaded liposomes (3 days vs 8 hr). This result suggested to the authors that the entrapped β-glucuronidase, although shown to be latent by the established criteria, must be partially exposed on the liposome surface and so provide a surface ligand that causes increased affinity for uptake by hepatic cells.

Even more revealing were the results of Steger and Desnick's (34) examination of the hepatic intra-

cellular localization of the administered bovine β-glucuronidase. Following administration of enzyme-loaded negative liposomes, 70% of the exogenous enzyme appeared in the lysosomal/mitochondrial fraction at times ranging from 1 to 144 hr postinjection. In contrast, the use of positive liposomes for enzyme delivery resulted in the appearance of only 50% of the exogenous enzyme in the lysosome-rich fraction at 1 hr postinjection and 20–30% from 1 to 4 days postinjection. In addition, four endogenous lysosomal enzymes were shown to undergo a concurrent translocation from the lysosomal fraction to the soluble fraction 1–3 days after administration of positive liposomes; by 5 days the intracellular distribution of lysosomal hydrolases appeared to return to normal. The authors suggest that the lysosomal membranes may have been destabilized by the positively charged stearylamine in the liposomal bilayers, resulting in the release of lysosomal contents into the cytoplasm.

The ability of invertase to express its catalytic function intracellularly was demonstrated previously in vitro (27). Cultured mouse peritoneal macrophages and Chinese hamster fibroblasts, both lacking endogenous invertase activity, were loaded with sucrose for 24 hr, resulting in a model lysosomal storage disease. Incubation of cells with invertase-containing liposomes resulted in the hydrolysis of stored sucrose to glucose and fructose and the subsequent disappearance of sucrose-laden cytoplasmic vacuoles.

Colley and Ryman (29, 30) created an in vivo model storage disease by injecting [^{3}H]dextran (mol wt 80,000) into rats in order to further prove the therapeutic usefulness of liposomes in enzyme replacement therapy. While hepatically-accumulated dextran was stable for up to 6 days, administration of liposome-entrapped dextranase or untrapped dextranase caused a precipitous 70% reduction within 2 days. Unfortunately, the latency of dextranase-containing liposomes in the blood has not been evaluated; the possibility exists that, within moments of injection, dextranase is released from liposomes and is independently taken up by the liver. In order to substantiate their claim, the authors must show that lipid phase markers, aqueous compartment markers, and dextranase are accumulated by the liver in the same proportions as exist in intact liposomes.

Morphologic analysis of liposomal distribution

The hepatic uptake of liposomes appears to be mediated initially by Kupffer cells, and secondarily by the parenchymal cells. DeBarsy and coworkers (72, 73) have shown by ultrastructural analysis that 1 hr after intravenous administration to newborn rats of liposomes, L(PC 4:SA 1:Chol 2), containing fluorescein-conjugated antibodies to lysosomal acid α-glucosidase, liposomes were free in the lumen of sinusoidal spaces and within the phagocytic vacuoles of macrophages and Kupffer cells. Only after 7.5 hr could liposomes be identified within parenchymal cells, at which time the Kupffer cells were grossly vacuolated and bordered on necrosis. In another investigation (74), electron microscopic examination of liver 2 hr after intravenous injection of liposomes, L(PC 7:PA 1:Chol 2), containing nitroblue tetrazolium revealed internalization of liposomes within the phagocytic vacuoles of Kupffer cells; no evidence was obtained for uptake by parenchymal hepatocytes. Wisse and Gregoriadis (25) similarly suggested that the initial uptake of horseradish peroxidase-loaded liposomes 3–30 min after intravenous infusion was by Kupffer cells. Uptake by parenchymal cells was first observed between 30 and 60 min and was most evident at 3 hr; liposomes were still present at 24 hr. In still another electron microscopic study of the liver, Rahman and Wright (75) observed endocytic uptake of EDTA-loaded liposomes by the Kupffer cells and parenchymal hepatocytes within minutes of infusion into mice; no evidence for liposome–cell fusion was obtained. Phagocytic vacuoles containing liposomes increased in size and number within the cytoplasm of both cell types for several hours; no attempt was made to quantitate the relative uptake by Kupffer cells and hepatocytes. These ultrastructural findings (25, 72–75) are further substantiated by cellular fractionation of the liver into populations of Kupffer cells and hepatocytes; 70% of the hepatic accumulation of liposome-entrapped [^{14}C]inulin was recovered in the Kupffer cell-rich fraction (76) 15 min after intravenous injection of liposomes L(PC 3:DCP 1:Chol 9). Tanaka et al. (76) calculate that Kupffer cells accumulate 5-fold more [^{14}C]inulin entrapped in liposomes than do parenchymal cells, on a per gram basis, assuming that Kupffer cells comprise 30% of the liver mass.

An early claim by Gregoriadis and Ryman (22) that 3 min after injection albumin-containing liposomes are present in parenchymal cells and "probably" in Kupffer cells has been challenged by the investigations cited above. Gregoriadis has more recently suggested (74) that his earlier observations based on radioautographic visualization of [^{3}H]cholesterol-labeled liposomes may have been biased by [^{3}H]cholesterol exchange between liposome and hepatocyte membranes. It appears not unlikely that Gregoriadis' early results (22) may reflect the effects of macro-

molecular surface ligands. The albumin-loaded liposomes may, to some extent, have had portions of the albumin molecules exposed on the outer liposome surface, thereby influencing in vivo tissue and cellular localization.

Clinical trials in man

In the first of two studies (77) in which liposomes were administered to human subjects, Gregoriadis and his colleagues investigated the fate of sonicated liposomes, L(DPPC 7:PA 1:Chol 2) [^{131}I-labeled albumin], in three patients with metastatic cancer. Patients received a single dose of either 37 or 7.5 mg of lipid by intraarterial or intravenous injection, respectively. The early time course, which was followed by using the hybrid-scanner technique, revealed a close correlation between hepatic accumulation and the rapid clearance of liposomal radioactivity from circulation; greater than 80% of the injected load was cleared within 6 hr. Tissue specimens, examined for accumulation of radioactivity, were obtained from one patient during surgery 3 hr after injection and from a second patient at autopsy 5 days after injection. Comparison of the accumulation by tumor tissue and "normal" tissue from the same organs revealed enhancement of uptake by tumor tissue; liver tumor tissue had 20–30% more accumulation than normal liver tissue, kidney tumor tissue had 50 times more accumulation than normal kidney tissue, and tumor tissue from spleen and colon had twice as much accumulation as their normal counterparts. The authors suggest that the enhanced uptake by tumor tissue may reflect either increased vascularization of the diseased tissue or the increased endocytic capacity of tumor cells. Finally, ultrastructural analysis of liver 3 hr after intravenous injection indicated uptake of liposomes by approximately one-third of the parenchymal cells; uptake by Kupffer cells was "extremely uncommon".

Recently, Tyrrell et al. (23) used liposomes, L(PC 7:PA 1:Chol 2), containing amyloglucosidase in an attempt to treat a patient with type II glycogenosis (Pompe's disease). The absence of lysosomal α-glucosidase leads to the accumulation of its substrate, glycogen, which in turn is reflected in clinical symptoms of muscular hypotonia, weakness, and finally death. Liposome-entrapped enzyme was intravenously injected each day for a week (total dose was 170 mg of lipid and 3 mg of enzyme); the patient died on the eighth day. The investigators note that the enlarged liver significantly decreased in size during therapy. At autopsy, although the liver glycogen level was half of that expected (on the basis of data accumulated for other children with Pompe's disease), glycogen storage by other tissues appeared to be unaltered by the enzyme replacement therapy. Trace amounts of administered α-glucosidase could be detected only in liver and spleen. The therapeutic usefulness of liposomes in the treatment of Pompe's disease is limited, as the authors point out, unless a technique is devised for the targeting of liposomes to muscle tissue.

Potential hazards involved in the use of liposomes

While liposomes are generally innocuous, substantial reason for concern has arisen regarding their use in enzyme replacement therapy. The two potential hazards inherent in their use in animals are the toxicity of the lipid constituents and the immunogenicity of the lipids and the entrapped proteins. Gregoriadis (78) warns of the toxicity of charged amphiphiles; intracerebral injections of liposomes into mice resulted in epileptic seizures and cerebral tissue necrosis when liposomes contained either stearylamine or dicetyl phosphate. Injection of high doses of dicetyl phosphate-containing liposomes into newborn mice resulted in death within 90 min; autopsy revealed petechiae and cardiac exudates (73). In addition, intravenous administration of sonicated phosphatidylserine liposomes to mice causes significant elevation of the free glucose levels in the blood and the brain tissue, probably triggered by catecholamine release by the adrenal glands (79). Recently, Steger and Desnick (34) have demonstrated that administration of positively-charged liposomes containing stearylamine, L(DPPC 7:SA 1:Chol 2), to mice resulted in disruption of lysosomal integrity in the liver whereas negatively charged liposomes, L(DPPC 7:PA 1:Chol 2), did not.

The toxicity of phosphatidylserine and stearylamine has also been demonstrated in vitro. Dunnick, Kallman, and Kriss (80) report that cultured tumor cells exposed to liposomes consisting of phosphatidylcholine, cholesterol, and gangliosides continued to proliferate normally whereas cells exposed to liposomes containing either phosphatidylserine or stearylamine became nonproliferative. These charged lipids are presumably incorporated into the cell membrane via liposome–cell lipid exchange and liposome–cell fusion and thereby exert adverse effects by altering essential membrane functions, such as the transport of nutrients. Similarly, Magee and coworkers (24) have observed that, when incubated with cultured HeLa cells at high doses, stearylamine-containing liposomes, L(SPH 3:SA 1:Chol 1.1), caused toxic effects, i.e., cells detached from the substratum and dis-

played severe bulging and cell membrane deformation.

In addition to the potentially adverse physiological consequences imposed by foreign lipids, there also exist immunological hazards. Enzymes entrapped within liposomes, although latent with respect to their catalytic activity, may have noncatalytic portions exposed on the outer surface of the liposome. This is strongly implied by the observation that β-glucuronidase-loaded liposomes display a totally different kinetics of hepatic accumulation and retention than buffer-loaded liposomes (34); presumptive exposed protein moieties act to direct the in vivo fate of the liposome. Proteins and other antigens entrapped in liposomes may be more immunoreactive and elicit the production of more antibody than the same dose of free protein or antigen (36, 81, 82). Glycolipid constituents of liposomes may also elicit antibody formation (58). Alving, Fowble, and Joseph (58) have shown that the immunogenicity of galactosyl lipids is directly related to the overall lengths of the molecules; ganglioside is strongly immunoreactive.

SUMMARY

The studies described above may be summarized as follows. *1*) Liposomes may be formed so as to encapsulate enzymatically active proteins or other macromolecules. *2*) The entrapped enzymes include many that are missing in lysosomal storage diseases. *3*) In vitro and in vivo administration of enzymes by means of liposomes increases the uptake by cellular lysosomes relative to the uptake of free enzyme. *4*) Targeting of liposomes containing enzyme to specific tissues is, at present, only minimally successful. *5*) Uptake by one or another tissue depends both upon the route of administration of liposomes (e.g., intravenous vs. intraperitoneal) and upon the composition of the liposomes (charge, size, content). *6*) The association of liposomes with cells may be enhanced by coating the liposomes with ligands recognizable by surface receptors. *7*) Problems of safety, toxicity, long-range effects, and doubts as to the exact subcellular localization of liposomes and their cargo are not yet resolved. This area of investigation is young and appears promising, but much work is required with experimental animals before it can be extended into the clinic.

Aided by grants (AM-11949, HL-19072, GM-23211, HL-19721) from the National Institutes of Health, the National Foundation–March of Dimes, the National Science Foundation (76-05621), the Whitehall Foundation, and The Arthritis Foundation.

Manuscript received 23 March 1977; accepted 23 September 1977.

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