Introduction of Purified Hexosaminidase A into Tay-Sachs Leukocytes by Means of Immunoglobulin-Coated Liposomes†

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ABSTRACT: To determine whether ligand–receptor interactions could engender the selective uptake by deficient cells of enzyme-laden liposomes, aggregated human IgG was used to coat liposomes which had previously trapped purified hexosaminidase A (Hex A). By a new, high-yield procedure, Hex A was purified 7000-fold from human placenta: the homogeneous protein had a p*I* of 5.4, permitting nonelectrostatic trapping in the aqueous interstices of anionic multilamellar liposomes (molar ratios of phosphatidylcholine–dicetyl phosphate–cholesterol, 7:2:1). Trapped Hex A was separated from free enzyme by means of Sephadex G-200 chromatography: 1.3 ± 0.3 mUnits of Hex A/μmol of phospholipid became associated with liposomes and trapped glucose, utilized as a marker of the aqueous compartment. Once sequestered, the enzyme remained latent until lamellae were disrupted by Triton X-100. Presence of enzyme in aqueous compartments was proved by the demonstration of increased trapping (0.02–1.33 mUnits/μmol of phospholipid) with increments in like-sign repulsion of the bilayers produced by increasing molar ratios of anionic dicetyl phosphate (5–20%). To provide for ligand–receptor interaction with surface Fc receptors of human polymorphonuclear leukocytes (PMN's), liposomes were coated by heat-aggregated (62 °C, 10 min) human IgG. PMN's from Tay-Sachs patients genetically deficient in Hex A activity readily incorporated exogenous Hex A provided in this fashion. PMN's exposed to enzyme-laden liposomes coated with aggregated IgG incorporated significantly more Hex A than when the enzyme was presented in uncoated liposomes or in liposomes coated with native IgG, which engages Fc receptors with less avidity. Free enzyme was *not* endocytized. Acquisition of specific Hex A isozyme activity by cells (determined by DEAE-cellulose chromatography) was not due to surface adsorption since cytochalasin B, which prevents phagocytosis but not surface adherence, blocked uptake. Incorporation of the isozyme by deficient cells was also demonstrated by starch gel electrophoresis, and ultrastructural studies showed that the immunoglobulin-coated, Hex A-containing liposomes were taken up into PMN lysosomes after membrane fusion. The studies indicate that liposomes coated with surface ligands may be used to introduce enzyme or other materials into deficient cells possessing appropriate surface receptors.

N-Acetyl-β-D-hexosaminidase (EC 3.2.1.52; hexosaminidase) catalyzes the hydrolysis of *N*-acetyl-β-D-galactosamine or *N*-acetyl-β-D-glucosamine residues from the nonreducing end of various substrates. By means of synthetic substrates, the enzyme in mammalian tissues can be separated into two major components, hexosaminidase A and hexosaminidase B (Srivastava et al., 1974a; Okada and O'Brien, 1969). The genetically determined absence of the lysosomal enzyme, hexosaminidase A, results in the massive accumulation of its natural substrate (GM_2-ganglioside) in lysosomes of patients with Tay-Sachs disease (variant B).

Since not only Tay-Sachs, but also Hunter, Hurler, and other storage diseases are caused by a genetic deficiency of specific acid hydrolases in lysosomes, reversal of this deficiency has been approached by means of direct enzyme replacement. Because it is the affected system, the lysosomal apparatus of cells, which 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 (Johnson et al., 1973). 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 ceramidetrihexosidase are infused (Brady et al., 1973, 1974). The problems associated with the direct administration of enzyme include (1) the inability of this method to direct the enzyme to parenchymal tissues containing the stored material, (2) the potential antigenicity of the enzyme, (3) the introduction of catalytic proteins into the circulation where they may interact with components of blood, and (4) inability of free enzyme to cross the blood-brain barrier.

In order to circumvent these problems, we have used liposomes as carriers of purified enzyme and have coated these enzyme-laden liposomes with an appropriate surface ligand for recognition and uptake. We prepare liposomes as a concentric bilayer of lipids alternating with aqueous compartment within which soluble substances such as enzymes can be entrapped (Bangham et al., 1965; Sessa and Weissmann, 1970), and have previously suggested that such liposomes can serve as vectors for the introduction of enzymes into genetically deficient cells (Weissmann et al., 1975).

When liposomes encounter the membranes of cells or organelles, they tend to resist uptake (Magee and Miller, 1972), provoke fusion of cells or organelles (Papahadjopoulos et al., 1973; Hawiger et al., 1969), or exchange their constituents with those of the cell surface, rendering their

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contents unavailable to the interior of the lysosomal apparatus (Huang and Pagano, 1975). This suggests that the phospholipid surface of liposomes does not constitute a strong endocytic stimulus. However, it has already been demonstrated that cells actively interiorize enzyme-containing liposomes when these are coated with a substance capable of engaging cell surface receptors (Weissmann et al., 1975). Lattices of aggregated human IgG adhere to polymorphonuclear leukocytes by attaching to the cells' surface Fc receptors (Henson et al., 1972), a process which triggers uptake either of the aggregated protein itself, or any particle coated with such aggregates (Weissmann et al., 1971).

In the present studies we have incorporated a homogeneous preparation of hexosaminidase A within liposomes and have found that its uptake by polymorphonuclear cells from patients with Tay-Sachs disease is significantly enhanced by prior coating of liposomes by aggregated, isologous IgG. Liposomes coated by this ligand were endocytized by the cells and the previously deficient cells acquired significant hexosaminidase A activity. The cells were not damaged in the phagocytic process as shown by their failure to leak cytoplasmic lactate dehydrogenase, and the enzyme-laden liposomes were localized to lysosomes by ultrastructural means. This method, which utilizes a specific surface ligand to key uptake in target tissues which possess appropriate surface receptors (e.g., the Fc receptor of the PMN) may prove to be of general use in the correction of genetic, lysosomal enzyme deficiencies.

Materials and Methods

Sources. Ovolecithin was obtained from Grand Island Biological Co., Grand Island, N.Y. Dicetyl phosphate was purchased from K & K Laboratories, Plainview, N.Y. Cholesterol was obtained from Fisher Scientific Company, Fair Lawn, N.J. Human placentas were obtained from maternity wards and stored at 4 °C for 1 to 24 h before use. 4-Methyllumbelliferyl-β-D-*N*-acetylglucosaminide was obtained from Pierce Chemical Company, Rockford, Ill. DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, England. Sephadex G-200, G-25, concanavalin A-Sepharose, and the columns used were obtained from Pharmacia, Uppsala, Sweden. Ecteola-cellulose was purchased from Sigma Chemical Company, St. Louis, Mo. Ampholine and electrofocusing equipment were obtained from LKB-Produkter AB, Bromma-A, Sweden. Dulbecco's phosphate-buffered saline was obtained from Grand Island Biologicals, Grand Island, N.Y. Rabbit antihuman whole serum antibody and rabbit antihuman IgG were purchased from Boehringer Corp., N.Y. Cytochalasin B was purchased from ICI Research Laboratories, Alderley Park, Cheshire, England. All the other reagents used were of analytical grade.

Purification of Hexosaminidase A. Hexosaminidase A was purified from human placentas. Hexosaminidase activity was determined by using 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide (Srivastava et al., 1974a). The placentas were washed with water, amnion and blood vessels were removed, and tissue was cut into small pieces, and homogenized with one part of water in a Waring blender for 2 min. Citrate-phosphate buffer (pH 4.5) was added to make a 30% homogenate and a final ionic strength of 0.015 M. It was further homogenized in a Sorvall omnimixer for 15 min at 8000 rpm. The homogenate was stirred overnight and centrifuged at 7000*g* for 15 min. The supernatant was fractionated and the precipitate was removed by centrifugation at 11 000*g* for 15 min at 4 °C. The precipitate was dissolved in a minimum amount of water and dialyzed overnight against two changes of water and lyophilized. The lyophilized powder was extracted three times with 200 ml of water and centrifuged at 29 000*g*.

Table I: Purification of Hexosaminidase A from Placenta.

Fractions	Total Protein (mg)	Enzyme Activity (units) Total	mg of Protein	Purification (fold)	Yield (%)
Homogenate supernatant	285 000	3420	0.012		100
$(NH_4)_2SO_4$	17 100	2565	0.15	12.5	75
Lyophilized extract	6 043	1390	0.23	19.2	41
Concanavalin A	206	778	3.78	315	23
G-200	59	739	12.57	1048	22
DE-52	13	495	38.12	3177	14
Isoelectric focusing and Ecteola	2	225	87.86	7322	7

The clear supernatant was then passed through a concanavalin A column (27 × 2 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7) containing 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 20 mM NaCl. When it was determined that the column was saturated with the enzyme, it was washed with 100 ml of equilibrating buffer containing 0.5 M NaCl. The elution of the enzyme was achieved with the buffer containing 1 M NaCl and 0.1 M methyl α-mannoside. The peak fractions were pooled and dialyzed against 10 mM Na/K phosphate buffer (pH 7.0) containing 100 mM $(NH_4)_2SO_4$. The dialyzed material was then pumped upwards through a Sephadex G-200 column (5 × 100 cm), equilibrated with dialyzing buffer, at a rate of 60 ml/h. The fractions containing the enzyme activity were pooled and dialyzed overnight against 10 mM potassium phosphate buffer (pH 6.0) with two changes and passed through a DEAE-cellulose (DE-52) column (2.5 × 40 cm) equilibrated with dialyzing buffer at a rate of 20 ml/h.

The column was washed with the equilibrating buffer and hexosaminidase A was eluted with a 2 l., 0–200 mM NaCl gradient in 10 mM potassium phosphate buffer (pH 6.0). The peak fractions were pooled and concentrated using an Amicon ultrafiltration apparatus using a PM 10 membrane (Amicon, Lexington, Mass.). This material was then dialyzed against 10 mM phosphate buffer and subjected to isoelectric focusing as described previously with 2.5% ampholine in the range pH 4–6 (Vesterberg and Svensson, 1966). After isoelectric focusing, the fractions containing enzyme activity were pooled and dialyzed against 10 mM phosphate buffer (pH 7.5) with several changes for 24 h. The dialyzed enzyme was passed through an Ecteola-cellulose column (1.5 × 25 cm) equilibrated with the dialyzing mixture at a rate of 20 ml/h. The column was washed with 100 ml of 10 mM phosphate buffer (pH 7.5) containing 50 mM NaCl. Hexosaminidase A was eluted from the column with a 1 l., 50–200 mM NaCl gradient in 10 mM phosphate (pH 7.5). The enzymatically active fractions from Ecteola-cellulose column were pooled and concentrated using an Amicon ultrafiltration cell. Analytical disc electrophoresis was done according to the method of Davis (1964).

Preparation of Liposomes. The preparation of liposomes has been described in detail before (Weissmann et al., 1975; Sessa and Weissmann, 1968, 1970; Sessa et al., 1969).

Briefly, for the preparation of liposomes, phosphatidylcholine, dicetyl phosphate, and cholesterol (molar ratio 7:2:1) were dissolved in chloroform and after rotary evaporation a uniformly thin lipid layer was formed. A 6-ml solution of hexosaminidase A, 0.5 unit/ml, in 0.29 M glucose was added to the round bottom flask containing the lipid film. After dispersing the lipid film by vortexing, the suspension (15 μmol of lipid/ml) was permitted to stand for 2 h. After swelling, the suspension was layered on top of a Sephadex G-200 column (2.5 × 40 cm). The liposomes were eluted with Dulbecco's PBS (pH 7.3) (15 ml/h). The effluents were collected in 3-ml samples and analyzed for lipid content (absorbancy at 520 nm), Hex A[1] (Srivastava et al., 1974a), and glucose (glucose oxidase method) (Sessa et al., 1969). Glucose and Hex A levels were assayed in liposome rich fractions after addition of 0.2% Triton X-100 (v/v).

Determination of Free and Latent Enzyme Activity. Hexosaminidase activity was assayed using 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide. One-tenth milliliter of the reaction mixture contained 20 μl of citrate–phosphate buffer (pH 4.5) (citric acid 0.1 M plus sufficient Na_2HPO_4, 0.2 M, to give a pH of 4.5); 10 μl of the sample. The mixture was incubated for 10 min at 37 °C. The reaction was stopped with 4 ml of 0.2 M glycine buffer (pH 10.7). The fluorescence was measured using an Eppendorf fluorimeter (Brinkman Instruments, Inc., Westbury, N.Y.); primary filter 313–366 nm, secondary filter 400–3000 nm. One unit of activity is that amount of enzyme which will liberate 1 μmol of substrate per minute at 37 °C and pH 4.5.

To determine latent Hex A activity, aliquots of the liposome peak were incubated with or without 0.2 ml of Triton X-100 (0.2% v/v) at room temperature. Incubation of liposomes with this concentration of Triton X-100 results in loss of their structural integrity and a concomitant release of the enzyme (Weissmann et al., 1966). Both the treated and untreated samples were rechromatographed on a Sephadex G-200 column (0.9 × 15 cm) and eluted with PBS (pH 7.3). One-milliliter samples were collected and were again assayed for content of liposomes, Hex A, and glucose.

Preparation of IgG and Coating of Liposomes. Saturated $(NH_4)_2SO_4$ was added to human serum and allowed to mix at 4 °C. After 30 min the preparation was centrifuged at 850*g* for 15 min. The precipitate was then dissolved in a minimum amount of saline and layered on a Sephadex G-25 column (2.5 × 40 cm, 20 ml/h) in 0.01 M phosphate buffer (pH 6.5). The protein peak was collected and mixed with packed DEAE-Sephadex A-50 for 30 min at 23 °C (Baumstark et al., 1964).

The mixture was filtered and washed with 0.01 M phosphate buffer (pH 6.5). The pH was adjusted to 7.3 with 1 M Na_2HPO_4 and concentrated with an Amicon ultrafiltration apparatus using a PM-10 membrane.

Protein was determined by the method of Lowry et al. (1951) using lysozyme as a standard. The product obtained was characterized by immunoelectrophoresis by using the method outlined by Scheidegger (1955). Arcs of precipitate were developed by the addition to the trough of rabbit antihuman whole serum antibody and rabbit antihuman IgG. The IgG collected was centrifuged in a Beckman model L-2 ultracentrifuge at 105 000*g* for 3 h. The supernatant (now free of aggregates) was termed "native IgG". Native IgG was next heat aggregated for 10 min at 62 °C. Liposome fractions (in phosphate-buffered saline (pH 7.3)) were incubated with 150 μg/ml of either native or aggregated IgG for 30 min at 37 °C (Weissmann et al., 1974).

Leukocyte Suspensions. Peripheral blood leukocytes from Tay-Sachs patients were obtained from freshly drawn, heparinized venous blood after sedimentation of the erythrocytes with 6% dextran. Contaminating erythrocytes were removed by hypotonic lysis. The leukocyte suspensions were washed and cells were resuspended in phosphate-buffered saline (pH 7.3). The osmolarity of the buffer was within 2% of 300 mosmol. Cells (ca. 85% PMN's) were counted in an ordinary hematocytometer.

Incubation of Liposomes with Tay-Sachs Phagocytes. After total and differential PMN counts were performed, the polymorphonuclear leukocyte concentrations were adjusted to 8 × 10^6/ml. Incubation tubes contained autologous active serum (0.1 ml), cell suspensions (0.5 ml), and liposomes (0.5 ml). Samples of L[Hex A, glucose], IgG.L-[Hex A, gluc], or AggIgG.L[Hex A, glucose] (see terminology) were prepared from the pooled peak liposome fractions (above). Free Hex A (12 mUnits, 0.5 ml) was added in some experiments at concentrations at least equivalent to those added to L[Hex A] and in some cases was incubated with AggIgG.L[Gluc]. To retard phagocytosis, cytochalasin B (5 μg/ml, originally in 1% dimethyl sulfoxide) was added at 37 °C for 10 min before incubation with liposomes. Tubes were incubated at 37 °C for 60 min in a shaking water bath, and centrifuged at 600*g* for 10 min. After two washes (600*g*, 10 min) in phosphate-buffered saline (pH 7.3), which sufficed to remove the bulk of free enzyme from the phagocytes (Weissmann and Rita, 1972), pellets were resuspended in distilled water (0.4 ml). Supernatants and pellets were assayed for Hex A, glucose, β-glucuronidase, and lactate dehydrogenase. (Triton X-100 (0.2% v/v) was added to some experimental tubes after incubation in order to disrupt both liposomes and cells for total enzyme determination.)

Enzyme Assays. β-Glucuronidase was determined after 18 h of incubation, using phenolphthalein glucuronidate (Weissmann et al., 1971) as substrate; absorbance was recorded at 540 nm in a Beckman DB spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.). Lactate dehydrogenase was determined by measuring reduction of NADP (340 nm) as previously described (Weissmann et al., 1971); readings were obtained every minute for 3 min at 340 nm in a Beckman DU spectrophotometer.

Electrophoresis. Horizontal starch gel electrophoresis was conducted using a Tris–citrate buffer system at pH 5.8 consisting of 0.22 M Tris and 0.086 M citric acid for the bridge buffer and 0.008 M Tris and 0.004 M citric acid for gel (Swallow et al., 1974). The gels were prepared containing 12% starch as described by Smithies (1955). Samples were inserted into the gels on small pieces of filter paper (Whatman No. 3 or No. 17) 9 cm from the cathodal end of the gel and electrophoresis was carried out using cooling plates at 4 °C for 5 h with a D.C. potential gradient of 13 V/cm.

The *N*-acetylhexosaminidase isozymes were located after

[1] This article utilizes a previously detailed system of notation (Weissmann et al., 1975) which permits shorthand description of the coating of liposomes and their capture of proteins or other solutes. All externally adherent materials are followed by a period as in "AggIgG." to indicate the coating of liposomes by aggregated immunoglobulin G. Next is written "L" for liposomes. All entrapped substances, such as proteins or small molecules, are next written in square brackets, such as [Hex A, glucose] to designate liposomes after capture of hexosaminidase A and glucose within aqueous compartments. PMN's, polymorphonuclear leukocytes; Hex A or Hex B, the A and B isozymes of hexosaminidase (EC 3.2.1.52), respectively.

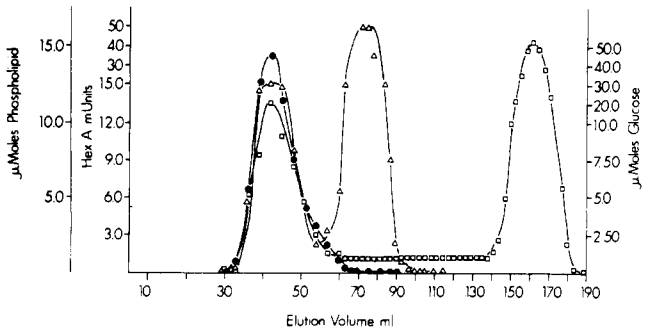

FIGURE 1: Separation of liposomes, hexosaminidase A (Hex A), and glucose on a Sephadex G-200 (2.5 × 40 cm) column. Liposomes, prepared with lecithin, dicetyl phosphate, and cholesterol in a molar ratio of 7:2:1 (15 μmol of lipid/ml) and swollen in glucose, 0.29 M, were mixed with a Hex A solution (0.5 unit/ml). The suspension was allowed to swell for 2 h, before chromatography. Elution was carried out with PBS and the effluent was collected in 3-ml samples. Lipid (●) was determined by apparent absorbance at 520 nm; glucose (□) was determined by the glucose oxidase method; Hex A (△) was determined by fluorometric measurement of methylumbelliferone E448.

electrophoresis by staining with the substrate, 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide, at a concentration of 0.5 mg/ml in citrate phosphate buffer (pH 4.5). Pieces of Whatman No. 3 or No. 17 filter paper cut to the size of the gel were soaked with freshly prepared substrate solution. The paper was placed over the gel and wrapped firmly around with saran wrap (Dow Chemical), and incubated at 37 °C. Areas of activity were visualized by viewing with long wave ultraviolet light.

Determination of Enzyme Activity in Cell Lysates. Cell pellets were treated with 0.2% Triton X-100 and sonicated (Ultrasonics Heat Systems, Inc., Plainview, N.Y.) for 2–20-s time periods, and layered on DE-52 columns (0.9 × 30 cm). The enzyme proteins were eluted with a linear gradient of 0–200 mM NaCl in sodium phosphate buffer, 10 mM, pH 6.0, 6 ml/h (Srivastava et al., 1974a). The effluents were collected in 1–2-ml samples and analyzed for hexosaminidase activity. The elution pattern of Hex A and Hex B by DE-52 column chromatography is presented in Figure 4a.

Electron Microscopy. Fixative was made by combining equal volumes of a stock solution of 2% acrolein and 3% glutaraldehyde in 0.1 M cacodylate buffer with 2% osmic acid in distilled water. The cell suspension was mixed with an equal volume of freshly prepared fixative and centrifuged (100*g* for 3 min). The supernatant was decanted and fresh fixative was added to the cells. Fixation time was 10 min. Cells were washed three times in physiological saline, stained with 1% uranyl acetate for 30 min, and dehydrated with ethanol. Spurr's low-molecular weight epoxy was used as the embedding medium. Sections were stained with lead citrate and viewed in a Zeiss EM 9S.

Results

Enzyme Homogeneity and Stability. Previous preparations of purified Hex A (Srivastava et al., 1974a) were obtained in too low a yield to permit bulk studies of the sort reported here. Consequently the methods previously described have been modified to yield 7% of total enzyme (Table I). Polyacrylamide disc electrophoresis of purified Hex A revealed a single protein band containing enzyme activity when stained with the substrate, 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide. The specific activity of the homogeneous preparation of Hex A was 87 U/ml protein (Table I). Repeated electrophoresis of Hex A over a 4-month period showed that the purified enzyme stored at 4 °C was very stable and no extra bands of protein were observed. The isolectric point of Hex A was 5.4 as determined by isoelectric focusing (see Materials and Methods).

Table II: Trapping of Hexosaminidase A by Liposomes Containing Increasing Amounts of Fixed Anion (Dicetyl Phosphate).

Liposome Composition in Molar Ratios of PC:DCP:Cholesterol	mUnits of Hex A/ μmol of Phospholipid[a]
85:5:10	0.02
80:10:10	0.28
70:20:10	1.38

[a] Hexosaminidase activity was determined after treatment with Triton X-100 (0.2% v/v).

Association of Hex A with Anionic Liposomes. A net negative charge was imposed upon liposomes by incorporation of dicetyl phosphate in the lipid mixture. When L[glucose] and Hex A were applied separately to the same column of Sephadex G-200, liposomes and glucose emerged after the void volume, whereas both free glucose and free enzyme were retained, as expected (Sessa and Weissmann, 1970). In contrast, when L[glucose, Hex A] was applied, liposomes were again eluted immediately after the void volume, followed by free Hex A and finally by untrapped glucose (Figure 1), but after eluates were incubated in the presence of Triton X-100 (see below), significant amounts of latent Hex A activity (and glucose) were shown to be associated with the liposome peak. In 12 experiments, 6.9% of recovered enzyme became associated with liposomes (1.3 × 0.3 munits/μmol of phospholipid). These experiments demonstrate that exclusion chromatography can resolve formed liposomes from free Hex A and that appreciable amounts of enzyme became associated with liposomes. As expected, addition of Triton X-100 did not increase the activity of Hex A emerging after the liposome peak.

Increments in Enzyme Trapping with Increments in Surface Charge. The formation of liposomes depends upon the capacity of the phospholipids to swell and form hydrated liquid crystals, consisting of a series of concentric bilayers which alternate with aqueous compartments. During swelling, liposomes can trap molecules within the aqueous spaces. The volume of these compartments is determined by the net charge of the lipids and the ionic strength of the environment (Bangham, 1968; Papahadjopoulos and Miller, 1967). As the net surface charge on liposomes increases, so does the interlamellar volume available for trapping; this property accounts for increments in solute trapping previously described (Weissmann et al., 1975; Sessa and Weissmann, 1970). When the molar percentage of dicetyl phosphate was increased from 5 to 10 to 20, increased amounts of anionic Hex A were trapped in the presence of negatively charged lipid lamellae (Table II).

Latency of L[Hex A, Gluc]. Control liposome fractions and fractions treated with Triton X-100 (obtained from pooled fractions by exclusion chromatography) were reapplied to Sephadex G-200 columns (Figure 2). On rechromatography, control liposomes retained the trapped enzyme and the glucose marker of their aqueous compartments within the lipid peak. In contrast, detergent-treated liposomes yielded up their previously trapped Hex A as well as

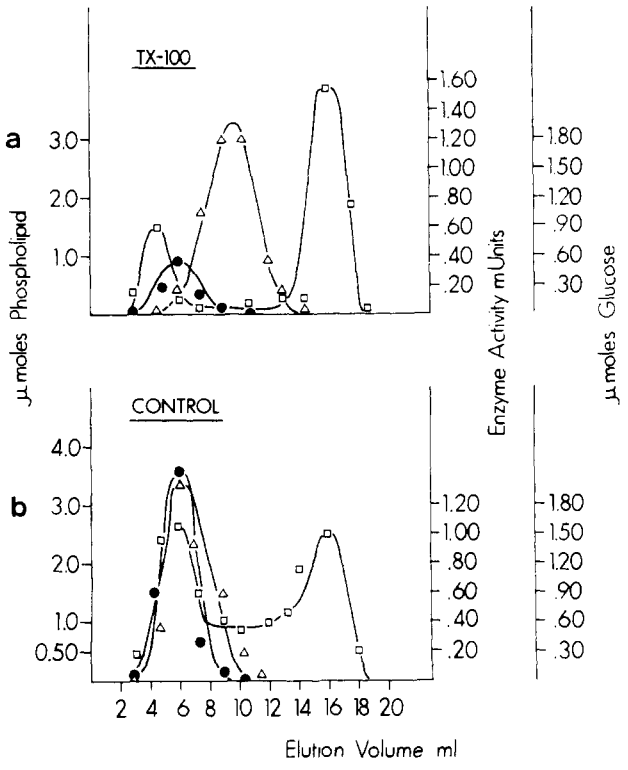

FIGURE 2: Aliquots of the liposome peak (Figure 1) were incubated with (a) or without (b) Triton X-100 (0.2% v/v). They were rechromatographed on a Sephadex G-200 column (0.9 × 15 cm) and eluted with PBS. Samples (0.5 ml) were collected and assayed for content of lipid, Hex A, and glucose. Lipid (●) was determined by apparent absorbance at 520 nm; glucose (□) was determined by the glucose oxidase method; Hex A (△) was determined by fluorometric measurement of methylumbelliferone E448. See Materials and Methods.

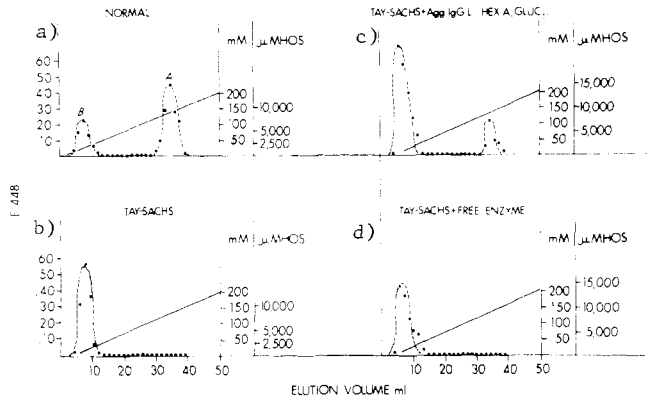

FIGURE 3: DEAE-cellulose (DE-52) column chromatography (0.9 × 30 cm) of cell lysates. The enzyme proteins were eluted from the column with a linear gradient of 0–200 mM NaCl in 10 mM phosphate buffer (pH 6.0). Lysates were treated with Triton X-100 (0.2% v/v) and sonicated for 2–20-s periods before being applied in the column. Effluents were collected in 1–2-ml fractions and Hex A activity (●- - -●) was determined by fluorometric measurement by methylumbelliferone cleaved from specific substrate, E448. (a) Isozyme distribution in PMN leukocytes of a normal subject. (b) Isozyme distribution in PMN leukocytes of Tay-Sachs patient distribution in PMN leukocytes of Tay-Sachs patient exposed to AggIgG.L[Hex A, Gluc] for 60 min at 37 °C. (d) Isozyme distribution in Tay-Sachs PMN leukocytes exposed to free Hex A in concentration 12 mUnits (equivalent to that added as L[Hex A]) for 60 min at 37 °C. See text.

glucose, which now emerged with elution patterns of the free enzyme or free glucose. Control L[Hex A] retained 82.2% of Hex A with the liposome peak. In contrast, detergent-treated L[Hex A] released 78.9% of trapped Hex A.

Homogeneity of IgG and Coating of Liposomes. The IgG obtained was characterized by immunoelectrophoresis and yielded a single line of precipitation with rabbit antibody to whole human serum and rabbit antihuman IgG.

The association of liposomes and aggregated IgG was previously shown to involve both electrostatic and hydrophobic interactions (Weissmann et al., 1974). We have previously shown by exclusion chromatography that 98% of heat-aggregated IgG became associated with anionic liposomes when 15 μg of aggIgG was used per micromole of phospholipid (Weissmann et al., 1974).

Uptake of Hexosaminidase A by Deficient Cells. Since enzyme assays of lysed cell pellets from patients with Tay-Sachs disease show no diminution in *total* enzyme activity as compared to normal leukocytes, the two isozymes (Hex A and B) were resolved by chromatography on DEAE-cellulose chromatography. Chromatography of lysed cell pellets of PMN's from normal subjects demonstrate that Hex A represented 63.5% of total hexosaminidase activity (Figure 3a). In contrast, no detectable amounts of Hex A were found in PMN leukocytes of Tay-Sachs patients (Figure 3b, Table III), although the usual, compensatory increment in Hex B activity was observed. Tay-Sachs leukocytes exposed to AggIgG.L[Hex A, Gluc] for 1 h demonstrated significant uptake of the enzyme after exposure to the particles at 37 °C (Figure 3c, Table III), and kinetic analysis (not shown) indicated 80% of maximum uptake by 30 min, with a plateau at 90 min. These cells, which had previously contained no detectable Hex A activity, now contained 15.8% of their total hexosaminidase activity as the A isozyme. Free enzyme was not incorporated by the cells, although the amount added was at least equal (12 mUnits) to that fed in the liposomes (Figure 3d). Tay-Sachs leukocytes exposed to IgG.L[Hex A, Gluc] incorporated significantly less ($p < 0.001$) enzyme than did cells exposed to AggIgG.L[Hex A, Gluc].

In order to exclude the possibility that liposomes coated with aggIgG did not induce the uptake of free enzyme (Table III) from the fluid phase by "piggyback endocytosis" (Sbarra et al., 1962), leukocytes were exposed to free enzyme (12 mUnits) and AggIgG.L[Gluc]. Lysates prepared from such cells showed only 1.9% of the Hex A isozyme (Table III).

To determine whether AggIgG.L[Hex A, Gluc] was truly phagocytosed rather than remaining adherent to the cell surface, cells were pretreated with cytochalasin B. This drug reversibly inhibits phagocytosis but does not interfere with surface adsorption (Zurier et al., 1973a). Lysates prepared from such cells yielded no detectable amounts of Hex A (Table III, Figure 4b).

Cells incubated with immunoglobulin coated liposomes as a phagocytic stimuli responded with the usual release of β-glucuronidase by "regurgitation during feeding" (Weissmann et al., 1971): 20.0 ± 2.9% release of total enzyme vs. 2.6 ± 0.24% of resting cells ($n = 6$). There was no evidence of cell death or cell membrane damage as shown by their failure to leak cytoplasmic lactate dehydrogenase into the supernates (Weissmann et al., 1971): 2.77 ± 0.29% of total enzyme vs. 1.5 ± 0.3% of resting cells ($n = 6$).

Starch Gel Electrophoresis. In order to resolve isozymes by an independent technique, lysates were also subjected to starch gel electrophoresis. In the first well (Figure 5), placental extract (used as the starting material for the purified

Table III: Hexosaminidase Activity in Lysed Cell Pellets of Human Polymorphonuclear Leukocytes.

Cells	Additions[a]	N	Total Hexosaminidase Activity (mUnits/10^7 Cells)[b]	Isozyme Distribution of Hexosaminidase (as Percent of Total)[b]	
				Hex A	Hex B
Normal		6	41	63.5	36.5
Tay-Sachs		6	42	0	100.0
Tay-Sachs	AggIgG.L[Hex A, glucose]	11	41	15.8	84.2
Tay-Sachs	AggIgG.L[Hex A, glucose] + cytochalasin B	4	33	0	100.0
Tay-Sachs	IgG.L[Hex A, glucose]	2	38	3.7	96.3
Tay-Sachs	L[Hex A, glucose]	3	38	3.7	96.3
Tay-Sachs	Free enzyme	4	41	0	100.0
Tay-Sachs	AggIgG.L[glucose] + free enzyme	2	34	2.0	98.0

[a] See footnote for abbreviations. Cells exposed to enzyme, liposomes, etc., 60 min, 37 °C. [b] Hexosaminidase activity in cell lysates was determined after treatment with Triton X-100 (0.2% v/v) and sonication for 2–20-s periods.

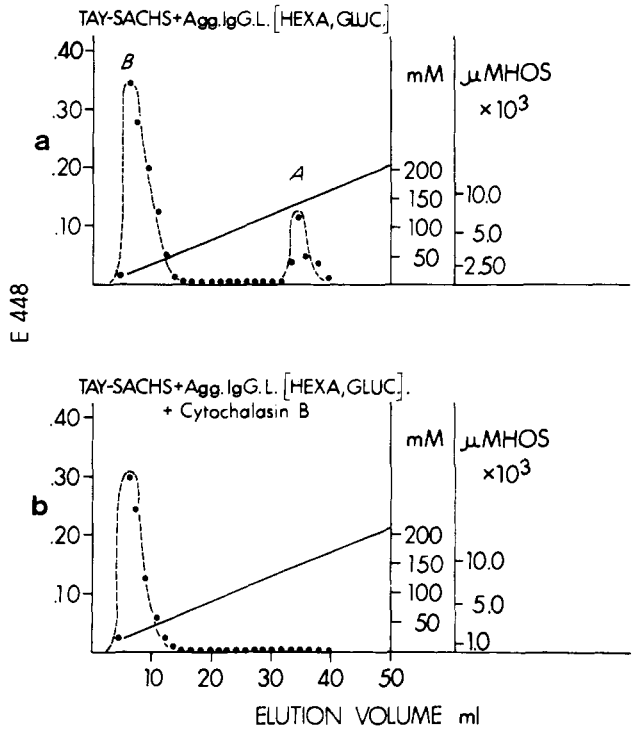

FIGURE 4: DEAE-cellulose (DE-52) column chromatography (0.9 × 30 cm) of cell lysates. Conditions as in Figure 3. Hex A activity (●- -●) distribution in PMN leukocytes of Tay-Sachs patient exposed to AggIgG.L[Hex A, Gluc] for 60 min at 37 °C. (b) Isozyme distribution of PMN leukocytes of Tay-Sachs patient pretreated with cytochalasin B, 10 min, 37 °C (to prevent phagocytosis) prior to exposure of cells to AggIgG.L[Hex A, Gluc].

Hex A) contained both major hexosaminidase isozymes: Hex B migrating to the cathode and Hex A migrating to the anode. In the second well, lysates of Tay-Sachs PMN's exhibited only Hex B activity. In the third and fourth wells lysates of PMN's from Tay-Sachs patients that had been exposed for 60 min to AggIgG.L[Hex A, Gluc] showed acquisition of new activity in the Hex A band. The fifth well contained Tay-Sachs cells treated with cytochalasin B before their exposure to AggIgG.L[Hex A, Gluc]; only Hex B activity was found. To exclude the trivial possibility of fluorescence of the reactants themselves (AggIgG.L[Gluc] in the absence of cells or enzyme), the sixth well contained these; no fluorescence was observed at either pole. The seventh well contained lysates of Tay-Sachs PMN's which had been exposed to free enzyme, demonstrating the presence only of Hex B.

Electron Microscopy. Ultrastructural studies of Tay-Sachs PMN's showed that uptake of aggIgG.L[Hex A, Gluc] was morphologically identical with phagocytosis of particulate stimuli by normal human PMN's. Pseudopods were extruded around single liposomes or clusters of liposomes and the plasma membrane beneath the liposome invaginated to form membrane-bound phagocytic vacuoles (Figure 6a,b). The cortical cytoplasm beneath the forming vacuole was filamentous and contained profiles of microtubules. Numerous microtubules were also seen in the centriolar region of these stimulated cells (Figure 6c). At later stages, profiles of fusion between liposome-containing phagosomes and PMN granules were common. PMN granule morphology was identical in Tay-Sachs and normal cells. Liposomes were present within 80–90% of PMN's examined after 60-min incubation with AggIgG.L[Hex A, Gluc]. A few monocytes also contained liposomes but none were seen associated with lymphocytes.

After processing for electron microscopy, most of the liposomes, both before and after phagocytosis, had the appearance of bubbles, 0.05–0.7 μ in diameter. The individual lamellae were closely appressed leaving an electron lucent space in the middle. Sometimes several smaller liposomes were seen within a larger one. In only a few, fortuitous sections were liposomes seen with evenly spaced, concentric lamellae.

Discussion

In order to proceed with experiments on the scale described here, it was necessary to obtain purified enzyme in good yield. It was possible to purify hexosaminidase A from human placenta 7000-fold (Table I), and the homogeneous protein proved stable for at least 4 months at 4 °C. In all its properties (Srivastava et al., 1974a,b), the enzyme obtained by the new procedure resembled that isolated by our previously reported, lower yield method (Srivastava et al., 1974a).

Once isolated, trapping of hexosaminidase in the aqueous spaces between the lipid lamellae of liposomes was demonstrated by the following experimental evidence.

(1) Exclusion chromatography clearly resolved Hex A associated with liposomes from the free enzyme: 6.9% of the recovered enzyme became associated with the lipid peak.

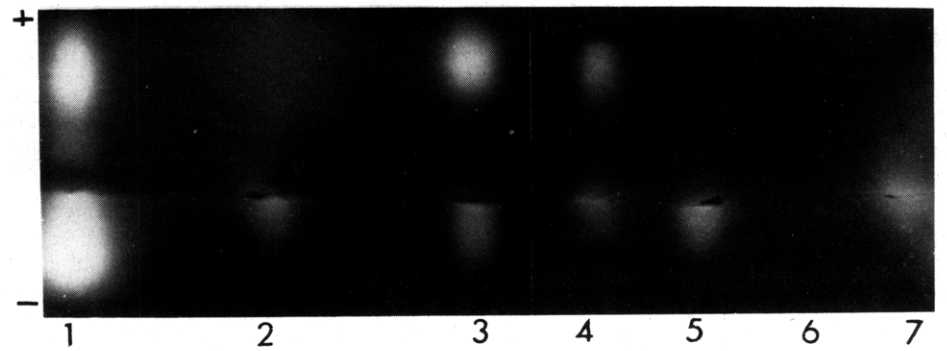

FIGURE 5: Horizontal starch gel electrophoresis of hexosaminidase isozymes using Tris–citrate buffer (pH 5.8). Hexosaminidase isozymes were located after electrophoresis by staining with the substrate, 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide (0.5 mg/ml in citrate–phosphate buffer pH 4.5, 37 °C): (1) placental extract; (2) Tay-Sachs leukocytes; (3–5) Tay-Sachs leukocytes + AggIgG.L[Hex A, Gluc] + cytochalasin B; (6) AggIgG.L[Gluc] in the absence of cells or enzyme; (7) Tay-Sachs leukocytes + free enzyme. The gel was incubated with the substrate at 37 °C + areas of activity were visualized by viewing with long wave uv light.

This is within the range previously reported for trapping of soluble enzymes such as lysozyme (15.1%) or peroxidase (5.8%) by liposomes of the like sign (Weissmann et al., 1975; Sessa and Weissmann, 1970). Moreover, the presence of trapped glucose in the liposome peak indicates integrity of the aqueous compartments.

(2) Latency of the trapped enzyme was demonstrated in that liposome-associated enzyme could only be assayed after the structural integrity of liposomes was disrupted by the nonionic detergent, Triton X-100. In contrast, free enzyme which was not liposome-associated could be assayed in the absence of detergent. Additionally, rechromatography of liposomes after detergent lysis showed that over 80% of trapped enzyme and glucose now chromatographed as free protein or sugar.

(3) As the net, anionic surface charge on the lamellae was increased by varying the molar percentage of dicetyl phosphate from 5 to 20, capture of Hex A was increased. Since only increments in the enclosed water space, due to like-sign repulsion of adjacent lipid layers in media of constant ionic strength, will produce increments of enzyme capture (Bangham, 1968; Papahadjopoulos and Miller, 1967), the anionic enzyme (p*I* = 5.4) is unlikely to have been trapped by nonspecific, electrostatic association.

In order to facilitate uptake of the liposome-encapsulated enzyme, the liposomes were coated with aggregated IgG. We have presented elsewhere in detail (Weissmann et al., 1974) proof that aggregated, rather than native Ig's preferentially coat, and partially insert into liposomes. At the concentrations used in these experiments (10 μg/μmol of phospholipid), over 98% of aggregated human IgG became associated with liposomes (prepared with 7:2:1 molar ratios of phosphatidylcholine–dicetyl phosphate–cholesterol) by both electrostatic and hydrophobic association (Weissmann et al., 1974). Since aggregated Ig's form lattices in which the key Fc regions are disposed both toward the interior of the outermost lamellae of the liposomes and also toward the surrounding medium, it would be expected that these would act as ligands for the Fc receptors of the PMN to provoke endocytosis (Weissmann et al., 1971).

In concordance with this hypothesis, Hex A was more actively taken up when presented to phagocytes as AggIgG.L-[Hex A, Gluc] than when presented in liposomes coated with native IgG or in uncoated liposomes. The efficiency with which particles are taken up depends upon the mechanism by which endocytosis proceeds. The kinetics of endocytosis have been examined and distinguished on the basis

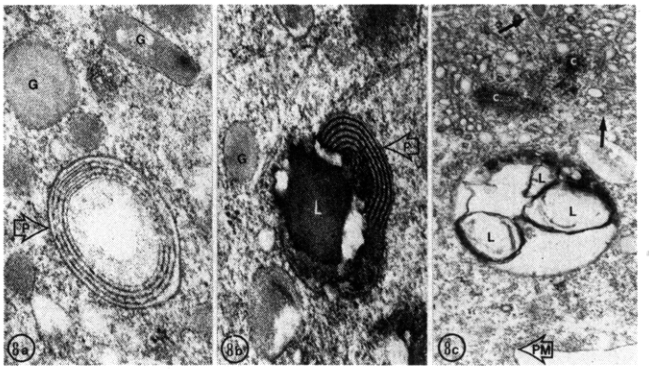

FIGURE 6: (a) Part of a Tay-Sachs PMN showing a liposome-containing phagosome (P) and characteristic cytoplasmic granules (G). Granule morphology is identical with that seen in normal human PMN's. Magnification 33 250×. (b) Part of a similar cell showing a phagosome (P) which contains a liposome fusing 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 6a 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×.

of surface receptor specificity (Jacques, 1969, deDuve et al., 1974). In *nonselective endocytosis,* solutes and small particles are taken up as a result of their presence in the extracellular fluid and the rate at which this substance is endocytized is a function of its concentration in the external medium (Jacques, 1969; deDuve et al., 1974). *Selective* endocytosis results when substances (actually: ligands) attach to specific membrane receptors. The uptake of Hex A observed in Tay-Sachs phagocytes in the presence of AggIgG.L[Hex A, Gluc] could be caused by two endocytic processes: (1) internalization of external fluid, i.e., nonselective endocytosis or (2) by internalization of the cell membrane which contains the receptor–ligand complex, i.e., selective endocytosis. But uptake of immunoglobulin-coated liposomes should proceed mainly via the latter process, due to the Fc receptor of the human PMN. This possibility has been tested.

The endocytic process has been characterized by the following equation (Jacques, 1969):

$$Q = Fc + SR\,[c/(K + c)]$$

where Q is the rate at which an extracellular substance is taken up; F is the volume of fluid taken up per unit time; c is the concentration of substance in the medium; S is the rate of engulfment of the plasma membrane; R is the surface density of receptor sites; and K is the ligand–receptor dissociation constant.

We have used the following three experimental conditions to demonstrate that virtually all of the Hex A incorporated by Tay-Sachs PMN leukocytes results from selective rather than from nonselective, or "piggyback", endocytosis.

In the *first,* free enzyme (12 mUnits) presented to cells engendered the uptake of 0 hexosaminidase A units/10 min. This indicates the absence of nonselective uptake without IgG's.

In the *second,* AggIgG.L[Gluc] with free enzyme (12 mUnits) engendered the uptake of 0.21 mUnit/10 min. This suggests, that, in the presence of Ig-coated particles, a minimum of free enzyme is carried in by "piggyback" phagocytosis as fluid, containing the free enzyme, is taken in along with the enzyme-free particles.

In the *third,* AggIgG.L[Hex A, Gluc] (12 mUnits) engendered the uptake of 2.5 mUnits/10 min. Now, the initial enzyme concentration was determined at the time of incubation. The amount of cellular uptake of Hex A, or Q, was determined by ion-exchange chromatography (Table III). The function, Fc, which represents nonselective endocytosis, equals zero in this system as demonstrated by a complete absence of Hex A incorporation in the first condition, thus

$$Q = SR\,[c/(K + c)]$$

The value for R is constant throughout the experiment because only Tay-Sachs PMN's were used. S and K remained constant under the second and third conditions in that the concentration of AggIgG coated liposomes was the same, thereby an equal inductive stimulus was provided both qualitatively and quantitatively. Thus, the observed difference in Q can only be accounted for by the concentration, c, of entrapped enzyme. These considerations suggest that selective endocytosis was the mode of entry.

Since uptake of immunoglobulin-coated liposomes proceeded in the presence of active serum, the role of complement deserves discussion. Although aggregated IgG might have been expected to activate complement via the classical pathway, and to lead to liposome disruption (Lachmann et al., 1970; Kinsky, 1972), this was not observed. There was no effect of AggIgG.L[Hex A, Gluc] on C-3, as judged by immunoelectrophoretic analysis (courtesy of Dr. I. Goldstein). Indeed earlier studies showed that uptake of immunoglobulin-coated liposomes by phagocytes of *Mustelus canis* (Weissmann et al., 1975) proceeds normally in heat-inactivated serum. Moreover, active complement is not an absolute requirement for endocytosis of immune complexes (Weissmann et al., 1971) nor for reverse endocytosis on immune complex coated surfaces (Zurier et al., 1973b). Since the Fc portion of the IgG molecule is the reacting ligand both with liposomes and cells (Henson et al., 1972; Weissmann et al., 1974), these studies suggest that complement is not activated because Clq binding is prevented by electrostatic interactions of the hinge region with anionic liposomes.

Previous studies by Gregoriadis, Ryman, and their coworkers (Gregoriadis and Ryman, 1972a,b) suggest that after injection of enzyme-containing, uncoated liposomes into rats, intact liposomes were readily removed from the circulation and subcellular fractionation of liver demonstrated that a crude mitochondrial/lysosomal fraction was the principal site of acquired enzyme activity (Gregoriadis and Ryman, 1972a,b). However, in contrast to our present studies, the mode of entry and the association of materials with cells remain uncertain. In the absence of controls for surface adsorption, it cannot be determined whether the injected material was interiorized or whether the liposomes did not simply adhere to the cell surface, subsequently to sediment with subcellular fractions. Since enzyme-laden liposomes were injected as long as a week after their preparation (Gregoriadis and Ryman, 1972a,b), it is possible that lipid–enzyme debris (formed in response to surface adsorption or fusion) may have become adherent to the surfaces of organelles during homogenization. Studies of cellular or lysosomal integrity were not reported, therefore, it is not possible to determine whether the enzyme–lipid complex produced injury to the reticuloendothelial cells which participated in their clearance. Our present study has excluded the possibility that enzymes were taken up by a nonselective process or after cell injury or death. The normal postphagocytic release of lysosomal β-glucuronidase was unaccompanied by leakage of cytoplasmic LDH. Ultrastructural morphology demonstrated uptake of liposomes in the lysosomes of phagocytes. No evidence for adherence or fusion with the plasma membrane was observed.

The present studies cannot, however, suggest a direct protocol for in vivo experiments at present. Although restitution of an in vitro deficiency has been achieved, many problems remain to be surmounted: proof of the safety of each of the reactants (most notably dicetyl phosphate and AggIgG adsorbed to liposomes) after intravenous or intraperitoneal administration, proof of lysosomal localization, determination of the fate and distribution of the vectors, and methods to force entry of leukocytes (bearing the ingested vectors) into affected tissues. Each of these is under study.

Finally, other workers have attempted to supply enzyme-deficient cells with purified lysosomal enzymes by other means. Purified enzymes have been injected directly into the circulation of patients with storage diseases or have been added to the media surrounding cultured fibroblasts (Brady et al., 1974; Porter et al., 1971; Wiesmann, 1974). In such experiments, the mechanism of endocytosis of lysosomal enzymes appears to be dependent on the nature of the oligosaccharide portion of the molecule and uptake can be influenced by a modification of this part of the glycoprotein (Morell et al., 1968; Hickman et al., 1974). *N*-acetyl-α-glucosaminidase, purified from human urine, is taken up by selective endocytosis in San Filippo B fibroblasts whereas the same enzyme, purified from human placenta, is taken up by nonselective endocytosis (VonFigura and Kresse, 1974; O'Brien et al., 1973). Since differences in electrophoretic mobility and antigenicity between Hex A from various human tissues have been attributed to differences in the sialic acid or carbohydrate content of the molecule (Gregoriadis, 1974), previous studies have suggested the use of a desialylated glycoprotein as a surface ligand for protein-containing liposomes (Gregoriadis, 1974). However, inconclusive results have been obtained from in vivo experiments which utilized [^{3}H]fetuin and its desialylated derivative to coat [^{125}I]albumin linked covalently to liposomes. Desialylated fetuin has an affinity for parenchymal cells of liver. After the animals were sacrificed, 80% of the injected ^{125}I was found to be concentrated in the liver when the fetuin–liposome complex was injected whereas 84% was localized

to liver after treatment with the desialylated derivative (Gregoriadis, 1974). This is the only other experiment reported in which the ligand–receptor model has been used to introduce liposomes into specific sites.

Theoretically, however, it would be most useful to provide the missing enzyme trapped in soluble form within liposomes, since the multicompartmental nature of this vector would protect the enzyme from the immune recognition sites in vivo, and hydrolytic attack within cells. Indeed, we have found that 30% of incorporated enzyme remained active intracellularly for 42 h. In the case of peroxidase-deficient dog fish cells, the enzyme remained completely active for 60 h. (Weissmann et al., 1975). Finally, the blood-brain barrier can be overcome by the normal (or pathologic) emigration of leukocytes: consequently, immunoglobulin-coated liposomes may eventually prove useful in vivo. We have no evidence, as yet, however, that once inside PMN lysosomes, Hex A can cleave its *natural* substrate (ganglioside GM_2), and studies directed toward this question are also in progress.

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