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A General Method for the Introduction of Enzymes, by Means of Immunoglobulin-Coated Liposomes, into Lysosomes of Deficient Cells

(immunoglobulins/horseradish peroxidase/storage diseases)

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ABSTRACT **Phagocytes of the smooth dogfish (*Mustelus canis*) contain no endogenous peroxidase within their lysosomes and constitute models for cells genetically deficient in lysosomal enzymes such as myeloperoxidase. We have obtained uptake of over 50% of exogenous horseradish peroxidase, provided the enzyme is exhibited to cells after incorporation into liposomes coated with heat-aggregated (62°, 10 min), isologous IgM. Trapping of horseradish peroxidase (EC 1.11.1.7) by liposomes was established by chromatographic resolution (Sephadex G-200; Sepharose 2B and 4B) of free enzyme from that associated with liposomes; liposome-associated horseradish peroxidase, together with trapped markers of the aqueous compartment (glucose, CrO_4^-), were excluded, and free enzyme and markers were retained. Enzyme and marker trapping was not electrostatic, varied with the molar ratio of charged membrane components, and was reversed by detergent lysis (Triton X-100) of liposomes. Uptake at 30° of aggregated IgM-coated liposomes containing trapped horseradish peroxidase exceeded that of free enzyme by 100-fold, and was more efficient than uptake of horseradish peroxidase presented in uncoated liposomes or in liposomes coated with native IgM. After phagocytosis, peroxidase-rich liposomes were localized exclusively in lysosomes of the phagocytes by ultrastructural histochemistry; the enzyme displayed over 50% latency to osmotic lysis. This method may prove to be of general use in the provision of exogenous enzymes to phagocytic cells genetically deficient in lysosomal hydrolases.**

We have previously demonstrated that lysozyme can be captured in aqueous spaces between the lipid lamellae of liposomes (1)*, and have suggested that liposomes can serve as vehicles for the introduction of enzymes into genetically deficient cells (5). Unfortunately, when liposomes encounter the membranes of cells or organelles, they tend to resist uptake (6), provoke fusion with cell membranes (7, 8), or remain adsorbed to cell surfaces where their contents remain unavailable to the cell interior (6). Although studies *in vivo* have shown uptake by rat liver and spleen of enzymes originally associated with injected liposomes (9, 10), no compelling evidence has been presented that enzymes captured by liposomes can be endocytosed by lysosomes of undamaged cells in the absence of surface adsorption or direct fusion.

Since it is doubtful that the phospholipid surfaces of liposomes constitute a strong endocytic stimulus to the lysosomal system (11), we reasoned that cells might actively ingest enzyme-containing liposomes if these were coated with aggregated immunoglobulin (12, 13). We have previously shown that leukocytes will endocytose immune precipitates in heat-inactivated serum (12), and that aggregated immunoglobulins preferentially associate with liposomes (13) by both electrostatic and hydrophobic interactions. Consequently, we have incorporated the enzyme horseradish peroxidase [HRP] (EC 1.11.1.7) within liposomes (L), and have found that uptake of L[HRP] by lysosomes of peroxidase-deficient phagocytes is significantly enhanced by prior coating of liposomes by aggregated, isologous, immunoglobulin.

As models of cells genetically deficient in lysosomal enzymes (14) we have used phagocytes of the smooth dogfish (*Mustelus canis*), which lack endogenous myeloperoxidase. We chose HRP for the following reasons: (*i*) the enzyme is only 2- to 3-fold larger (molecular weight 40,000; ref. 15) than lysozyme, which had previously been incorporated into liposomes (1); (*ii*) the sensitivity of HRP assay (<1 ng/ml) (15); (*iii*) the relative lack of uptake of free enzyme by phagocytes (16); (*iv*) HRP is anionic at pH 7.4 (pI = 7.2), which renders anionic liposomes useful for demonstrating nonelectrostatic trapping (1, 13); (*v*) the sensitivity of ultrastructural localization by histochemistry (17); and finally, (*vi*) because peroxidase deficiency in man is a defined genetic disease of lysosomes (18).

MATERIALS AND METHODS

Preparation of Liposomes. Liposomes and the sources of lipids and other materials have been extensively described (1, 13, 19). "Swelling solution" was prepared by dissolving 400 μg of HRP (Boehringer) in 6 ml of 0.29 M glucose to

* The term "liposome," first proposed in 1968 (2), has proved to be generally acceptable (3). This article utilizes a new, shorthand system of notation. All externally adherent materials will be followed by a period, as in "aggIgM." Next is written "L" for liposomes, followed by the molar ratios of membrane lipids, as in L(PC 7:DCP 2:Chol 1) enclosed in parentheses, to indicate phosphatidyl choline, dicetylphosphate, and cholesterol in the ratios indicated, respectively. All entrapped substances, such as proteins or small molecules, are next written in square brackets, such as [HRP, glucose] to designate liposomes after capture of horseradish peroxidase and glucose within aqueous compartments. Proteins such as ATPase (4) actually incorporated in lipid bilayers also belong between the parentheses, e.g., L(PC 7:DCP 2:Chol 1, ATPase). Consequently, liposomes chiefly described in the present experiments can be completely noted as: aggIgM.L(PC 7:DCP 2:Chol 1)[HRP, glucose], or, when the lipid composition is not at issue, as: aggIgM.L[HRP, glucose].

which 0.05 M phosphate buffer (pH 7.4) had been added, or by dissolving 400 μg of HRP in K_2CrO_4 (0.145 M, pH 8.15). Liposomes (15.0 μmol of lipid per ml) were permitted to capture HRP and markers of the aqueous compartment (glucose, $CrO_4^=$) by swelling for 2 hr (23°). Assays of HRP both before and after swelling showed no loss of activity provided pH was above 7.2. HRP, glucose, and/or $CrO_4^=$ associated with liposomes ([HRP], [glucose], [$CrO_4^=$]) were resolved from "free" enzyme or markers by means of exclusion chromatography on Sephadex G-200, Sepharose 2B, and Sepharose 4B (Pharmacia, Stockholm). Aliquots of liposomes (5 ml) were applied to 2.5 × 20-cm columns (operating pressure: 20 cm of water) with a flow rate of 0.1–0.5 ml/min; elution was in 0.145 M NaCl–KCl (equimolar) containing 0.05 M phosphate buffer (pH 7.4). Column fractions (1–3 ml) were analyzed for peroxidase as described by Steinman and Cohn (16); they were analyzed for liposomes (apparent absorbance at 410 nm) and for glucose (glucose oxidase method) or $CrO_4^=$ (absorbancy at 370 nm) as described (1, 13, 19). [Glucose] and [$CrO_4^=$] were assayed in liposome-rich fractions after addition of 0.2% Triton X-100 (v/v).

Studies of Liposomal Latency. Aliquots (0.5 ml of each liposome-associated peak: L[HRP, glucose] or L[HRP, $CrO_4^=$]) eluted from Sephadex G-200, Sepharose 2B, or Sepharose 4B were incubated at 37° for 30 min with or without 0.2% (v/v) Triton X-100. They were next rechromatographed on a small (0.75 x 5.5 cm) Sephadex column with buffered NaCl–KCl. Eluates (1.0 ml) were again assayed for content of liposomes, HRP, and glucose or $CrO_4^=$.

Incubation of Liposomes with Mustelus canis Phagocytes. Blood from caudal veins of dogfish was dispensed (10 ml) into heparinized (50 IU/ml) plastic tubes. Buffy coats were sedimented at 600 × *g* for 10 min; pellets contained 22.0 ± 3 (n = 6) percent phagocytes (supravital staining with toluidine blue). Cells were aspirated in pasteur pipettes, washed, and resuspended in inactivated (56°, 30 min) isologous plasma to a final concentration (hemocytometer) of 5×10^7 phagocytes per ml. Incubation tubes contained inactive plasma (2.0 ml), elasmobranch Ringer's solution (MBL Formulary, 900 mosm, pH 7.4, 1.5 ml), cell suspensions (0.5 ml), and liposomes (0.5 ml). Samples of L[HRP, glucose], aggIgM.L[HRP, glucose], or IgM.L[HRP, glucose] were prepared from the pooled, peak liposome fractions obtained by exclusion chromatography. Similar experiments were performed with L[HRP, $CrO_4^=$], aggIgM.L[HRP, $CrO_4^=$], and IgM.L(HRP, $CrO_4^=$]. Free HRP (0.5 ml) was added in some experiments at concentrations at least equivalent to those added as [HRP], while to others, Triton X-100 (0.2%, v/v) was added in order to disrupt both liposomes and cells for total enzyme and protein determination. To retard phagocytosis, cytochalasin B (5 μg/ml, originally in 1% dimethylsulfoxide) was added at 30° for 10 min before incubation with liposomes. Tubes were incubated either at 30° or 4° in a shaking water bath and centrifuged at 600 × *g* for 10 min. After two washes (600 × *g*, 10 min) in elasmobranch Ringer's solution, which sufficed to remove the bulk of free peroxidase from phagocytes (19), pellets were resuspended in distilled H_2O (1.0 ml). Both supernatants and pellets were assayed for HRP, glucose, β-glucuronidase (12), and lactate dehydrogenase (12); samples containing Triton X-100 were analyzed for protein (Lowry).

Latency of [HRP] in Phagocytes. Following the earliest protocol of deDuve (11), aliquots of cell pellets (0.1 ml) in H_2O were assayed several times over a period of 60 hr for [HRP]. Increments in enzyme activity were interpreted as reflecting latency of [HRP] within phagocytes.

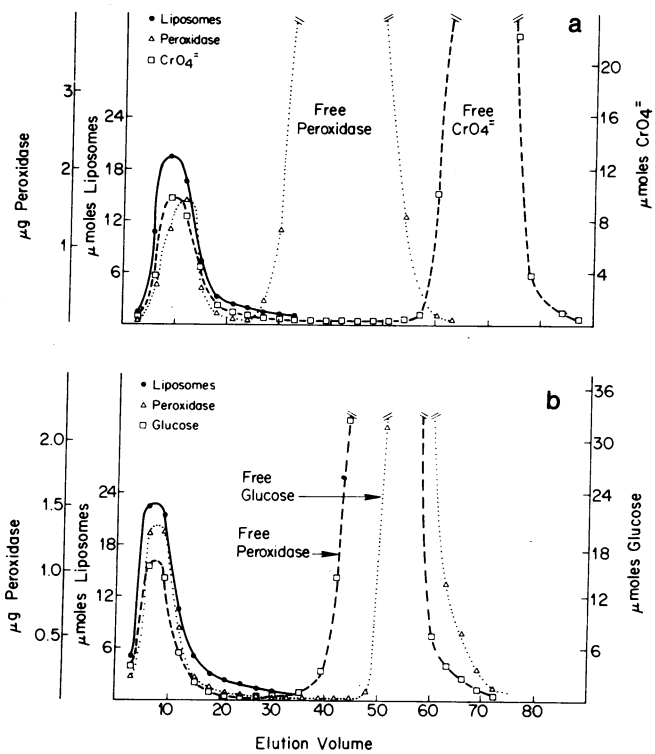

FIG. 1. (a) Sephadex G-200 chromatography of liposomes (●), horseradish peroxidase (△), and $CrO_4^=$ (□). Liposomes (15.0 μmol of lipid per ml) were prepared with phosphatidylcholine, dicetylphosphate, and cholesterol in molar ratios of 7:2:1. Liposomes were permitted to swell for 2 hr with 400 μg of HRP in 0.145 M K_2CrO_4 (pH 8.15) before they were eluted in 0.145 M NaCl–KCl, 0.05 M phosphate buffer (pH 7.4). (b) Sepharose 2B chromatography (2.5 × 20-cm column) of liposomes (●), HRP (△), and glucose (□). Liposomes (as above) were permitted to swell with 400 μg of HRP in 0.29 M glucose, 0.05 M phosphate buffer (pH 7.4). Elution was in buffered saline as above. For analytical procedures, see *Materials and Methods*.

Electron Microscopy. Cell pellets were fixed in 2% glutaraldehyde and 0.6 M sucrose in 0.1 M cacodylate buffer (pH 7.2) for 1 hr at 4°. They were washed four times in cold 11% sucrose in 0.2 M cacodylate buffer and incubated 120 min at room temperature in Graham and Karnovsky's medium (16) for peroxidase. Controls were without H_2O_2 or without 3′,3′-diaminobenzidine. Subsequent processing for electron microscopy was as described (21, 22).

Preparation of IgM and Coating of Liposomes. Elasmobranchs possess 19S and 7S immunoglobulins of the IgM class (23). 19S IgM of *Mustelus canis* was prepared by Clem and Small's procedure (23). The IgM pool (Sephadex G-200 chromatography after euglobulin precipitation) was centrifuged in a Beckman model L-2 ultracentrifuge at 105,000 × *g* for 1 hr. The supernatant (now free of aggregates) was termed "native IgM," and yielded a single line of precipitation by immunoelectrophoresis with purified rabbit antibody against dogfish IgM (courtesy of Dr. L. W. Clem). Native IgM was next heat-aggregated for 10 min at 62°, and both "native"

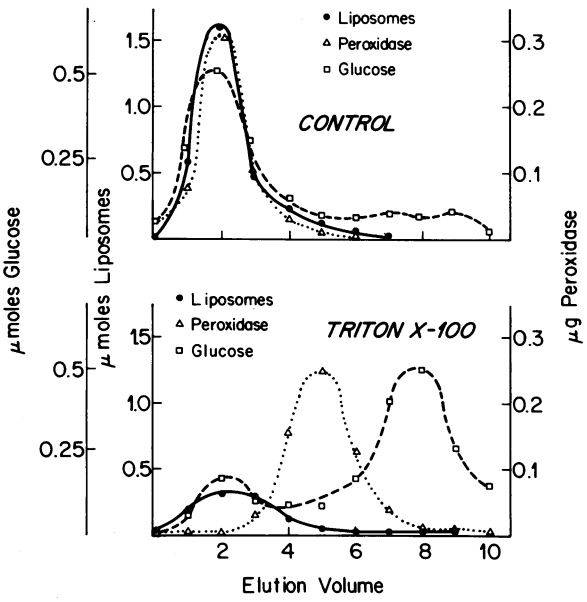

FIG. 2. Sephadex G-200 chromatography (0.75 × 5.5-cm columns) of liposome peaks after incubation (37°, 1 hr) with and without Triton X-100 (0.2%, v/v) to demonstrate latency. Pooled liposome fractions (Fig. 1b and Table 1) (1.0 ml) eluted in 0.145 M NaCl–KCl, 0.05 M phosphate buffer (pH 7.4). Liposomes (●); HRP (△); glucose (□).

and "aggregated" IgMs were analyzed (n = 3) in a Spinco model E analytical centrifuge. "Native" samples contained one sharp peak sedimenting at $s_{20°,w}$ of about 19S, with a minor (5–7%) component at 28S; "aggregated" samples contained multiple peaks with sedimentation properties greater than dimers of the 19S material. Liposome fractions (in 0.145 M NaCl–KCl, pH 7.4) were incubated with 150 μg/ml of either "native" or "aggregated" IgM for 30 min at 18°.

RESULTS

Association of [HRP] with Liposomes by Gel Filtration. Exclusion chromatography in three systems (Sephadex G-200, Sepharose 2B, and Sepharose 4B) clearly resolved enzyme

TABLE 1. *Association of peroxidase and glucose with liposomes by exclusion chromatography*

Column (n)	μmol of glucose (G) or $CrO_4^=$/μmol of phospholipid	ng of peroxidase/μmol of phospholipid	Percent associated*	
			Glucose or $CrO_4^=$	Peroxidase
Sephadex G-200 (3)	0.55 ($CrO_4^=$)	162	6.2	3.1
Sepharose 2B (4)	0.53 (G)	120	18.2(G)	7.5
Sepharose 4B (2)	0.90 (G)	593	24.0(G)	4.3
Mean: All columns (9)	0.67	313	14.2	5.8

* Percent of total anions or glucose associated with liposome peak (see Fig. 1). Liposomes were prepared with phosphatidylcholine:dicetylphosphate:cholesterol in ratio of 7:2:1.

TABLE 2. *Trapping of glucose and peroxidase by liposomes containing increasing amounts of fixed anion (dicetylphosphate)*

Liposome composition in molar ratios of PC:DCP:cholesterol	μmol of glucose/μmol of phospholipid*	ng of peroxidase/μmol of phospholipid*
9:0:1	0.041	20
8:1:1	0.450	160
7:2:1	0.900	593

* Associated with liposome peak of Sepharose 4B chromatographs. See *Materials and Methods* for details.

associated with liposomes [HRP] from free HRP (Fig. 1a and b). A summary of all columns is shown in Table 1. Approximately 6% of recovered enzyme became associated with liposomes: an amount comparable to previously trapped enzymes (1, 10).

Increments in Enzyme Trapping with Increments in Surface Charge. As the net surface charge on liposomes increases, so does the interlamellar volume (V_{H_2O}) available for trapping (1–3). When the molar percentage of dicetylphosphate (DCP) was increased from 0 to 10 to 20 (Table 2), increments of glucose and HRP trapping were observed. In contrast, cationic liposomes [L(PC 7:SA 2:Chol 1)] formed precipitates with HRP at pH 7.4. Glucose trapping (Table 2) did not increase as much as that of HRP in the presence of 20% DCP, presumably because HRP (molecular weight 40,000) excluded water between some lamellae.

"Latency" of [HRP, Glucose] or [HRP, $CrO_4^=$]. Control liposome fractions and fractions treated with Triton X-100 (obtained from pooled fractions obtained by exclusion chromatography) were reapplied to a mini-column of Sephadex G-200 (Fig. 2). Detergent-treated liposomes yielded up their previously trapped [HRP] as well as [glucose] and [$CrO_4^=$], which now emerged with elution patterns of the free enzyme or marker molecules. When formed with [HRP, glucose], control liposomes (n = 3) retained a mean of 98% of enzyme or glucose with liposome peaks; those formed with [HRP, $CrO_4^=$] (n = 3, not shown) retained identical amounts. In contrast, detergent-treated L[HRP, glucose] or L[HRP, $CrO_4^=$] released 61% of trapped peroxidase, 77.5% of trapped glucose, and 60.5% of trapped $CrO_4^=$.

Uptake of aggIgM.L[HRP, Glucose] by Mustelus canis Phagocytes. Phagocytes exposed to aggIgM.L[HRP, glucose] incorporated significant amounts of HRP as early as 15 min after exposure to the particles at 30° (Fig. 3). The cells of this poikilotherm also incorporated the HRP at 4°, but at a lesser rate; free enzyme was not incorporated to any appreciable extent (Fig. 3 and Table 3), although the amount added was at least equal to that fed in liposomes. Uncoated liposomes, L[HRP, glucose], or those coated with "native" immunoglobulins, IgM.L[HRP, glucose], took up significantly less enzyme than did cells exposed to aggIgM.L[HRP, glucose]. When phagocytosis was retarded (21) by incubation of the cells in the presence of cytochalasin B or at 4°, less HRP appeared associated with cell pellets; combining these inhibitory conditions almost completely prevented uptake of HRP (Table 3). Cells exposed to aggIgM.L[HRP, glucose] ingested glucose as well as HRP (the cells contain no endogenous free glucose), to the extent of 2 nmol/10^6 cells per hr

TABLE 3. *Uptake of peroxidase by, and release of enzymes from, phagocytes of Mustelus canis*

Cells incubated with*	n	ng of HRP/10^6 cells per hr taken up†	% β-Glucuronidase released‡	Lactate dehydrogenase % released§
HRP (900 ng added)				
30°	12	0.08 ± 0.001	5.2	0.05
L[HRP, glucose] 30°	6	4.1 ± 0.2	4.7	0.1
IgM.L[HRP, glucose]				
30°	12	4.8 ± 0.3	5.8	0.2
aggIgM.L[HRP, glucose]				
30°	12	9.7 ± 0.5	11.2	0.4
4°	4	5.7 ± 0.3	2.4	0.3
+ cytochalasin B, 30°	4	5.5 ± 0.7	18.1	0.4
+ cytochalasin B, 4°	4	2.2 ± 0.6	2.1	0.7

* See footnote on p. 88 for nomenclature.

† Mean ± SEM ng of HRP (assayed after 60 hr to permit full expression of latency) calculated from 0- to 15-min initial uptake rates. Mean amount of [HRP] trapped in added liposome suspensions = 485 ng per 2.5 × 10^7 phagocytes, or > 50% maximum uptake.

‡ Enzyme activity released from cells into supernatants (plasma blank subtracted), calculated as percentage released at 60 min by 0.2% (v/v) Triton X-100. Means of three experiments.

§ Calculated as for β-glucuronidase. Means of five experiments.

(mean, n = 6), whereas those exposed to L[HRP, glucose] incorporated only 0.54 nmol/10^6 cells per hr (mean, n = 6, not shown in Table 3). Glucose was assayed at 30 and 60 min.

Cells incubated with coated liposomes as phagocytic stimuli responded with the usual selective release of lysosomal β-glucuronidase by "regurgitation during feeding" (12, 20–23) (Table 3) without evidence of cell death or plasma membrane damage, as shown by the absence of cytoplasmic lactate dehydrogenase release into supernatants (12, 20–23). Cells exposed to cytochalasin B secreted enhanced amounts of the lysosomal enzyme by the mechanism of "reverse endocytosis" (22).

Electron Microscopic Observations. Ultrastructural observations showed that of cells examined that were exposed to aggIgM.L[HRP, glucose], only phagocytes (22% of cells) had endocytosed liposomes. One or more lysosomes in virtually all such cells contained HRP liposomes, which had the appearance of myelin figures associated with electron-dense peroxidase reaction product (Fig. 4a) and which were not observed in controls. A few cells were seen with peroxidase-positive phagosomes that were in the process of fusing when fixed, as seen in Fig. 4b. *No* evidence of liposome/cell membrane fusion was observed. Cells from suspensions incubated with an equal concentration of free HRP showed no peroxidase reaction product within their lysosomes (Fig. 4c).

Latency of HRP in Phagocytes. HRP activity of cell pellets suspended in distilled H_2O was determined as a function of time (11). Increments of HRP activity were observed to reach maximum values by 60 hr of standing at 4° (Fig. 5), suggesting that compartmentalization of HRP was gradually overcome in H_2O lysates.

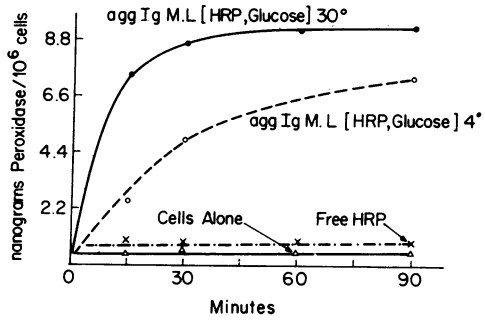

FIG. 3. Uptake of HRP by phagocytes of *Mustelus canis*. Phagocytes were incubated for times indicated with liposomes coated by aggregated, isologous IgM after capture of HRP in glucose (aggIgM.L[HRP, glucose]) at either 30° (●) or 4° (○) [liposomes were obtained from excluded peaks of Sepharose 2B chromatography (Fig. 1b) containing 400 ng of HRP per 2.5 × 10^7 phagocytes] or with 800 ng of free enzyme (×). Cells alone (△). After cell pellets were washed (see *Materials and Methods*), they were permitted to stand at 4° for 60 hr to permit full expression of latent enzyme, and HRP was then assayed.

DISCUSSION

Four lines of evidence suggest that HRP is trapped *between* lamellae of liposomes before their presentation to cells: First, liposome-associated HRP emerged with excluded volumes in three chromatographic systems, eluting together with markers of the aqueous compartment (glucose, $CrO_4^=$). In contrast, uncaptured glucose, $CrO_4^=$, and HRP were retained. Second, anionic enzyme was captured by anionic liposomes, e.g., L(PC 8:DCP 1:Chol 1), whereas cationic liposomes, L(PC 8:SA 1:Chol 1) formed precipitates with HRP in media of low dielectric constant (0.29 M glucose). Third, as the surface charge of lamellae was increased by increments in the molar percentage of charged component (DCP) from 0 to 20, capture of both HRP and glucose increased. The obverse would hold were simple electrostatic or hydrophobic interactions to induce surface coating (1). Finally, latency of trapped HRP, glucose, and $CrO_4^=$ was demonstrated after rupture of the lamellae by the nonionic detergent Triton X-100, which is further evidence against electrostatic association.

Once captured, HRP was more avidly taken up when presented to phagocytes as aggIgM.L[HRP, glucose] than when

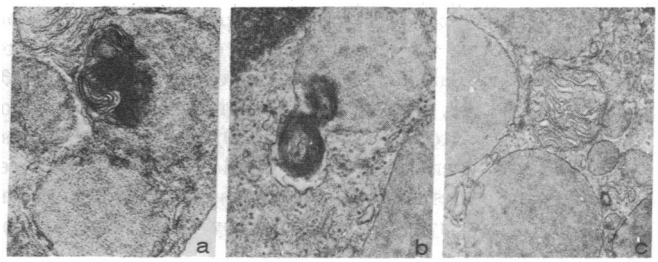

FIG. 4. (a) A portion of a phagocytic cell showing three lysosomes, one of which contains aggIgM.L[HRP, glucose]. Peroxidase reaction product is prominent in and around the liposomes but not in the remainder of the lysosome. Magnification, ×25,380. (b) A phagosome containing a liposome appears to be fusing with a lysosome. Magnification, ×25,380. (c) A portion of a phagocytic cell from a control cell suspension exposed to soluble HRP. No peroxidase-positive myelin figures are visible. Magnification, ×25,380.

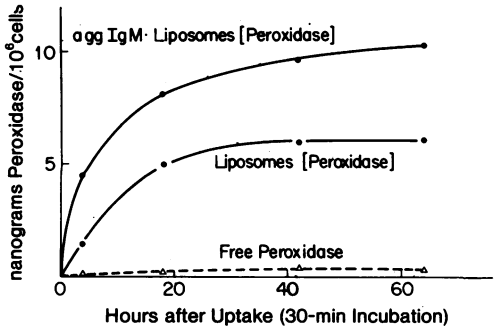

FIG. 5. Latency of HRP after uptake by phagocytes of *Mustelus canis*. After 30 min of incubation in the presence of liposomes containing HRP (500 ng/2.5 × 10^7 phagocytes) coated with aggregated IgM, of uncoated liposomes containing HRP (500 ng/2.5 × 10^7 phagocytes), or of free enzyme (800 ng/2.5 × 10^7 phagocytes), cell pellets were resuspended in distilled water and HRP activity was assayed to show the effect of osmotic lysis.

presented in liposomes coated with "native" IgM or in uncoated liposomes. Clear differences observed between the uptake by cells of aggIgM.L[HRP, glucose] and that of IgM.L-[HRP, glucose], together with physical differences between aggregated and native IgMs observed in the analytical ultracentrifuge, suggest that the critical factor for enhancement of uptake is heat aggregation at 62° for 10 min. Indeed, during the first hour, over 50% of added enzyme was incorporated by phagocytes when presented with aggIgM.L[HRP, glucose], in contrast to less than 1% taken up of free enzyme. Active complement is not an absolute requirement for endocytosis of immune complexes (12) nor for reverse endocytosis on immune complex coated surfaces (20); it was not necessary for uptake of aggIgM.L[HRP, glucose].

In previous studies, Gregoriadis and Ryman (9, 10) have followed the fate of L[(3H)amyloglucosidase] and L[invertase] after intravenous injection in rats and demonstrated association of label and/or enzyme (invertase) with sedimentable fractions of liver. However, the mode of entry and association of these materials with cells remains entirely obscure; in the absence of ultrastructural cytochemistry it cannot be determined whether the injected materials were ingested or whether they did not simply adhere to the cell surface or to organelles after homogenization. In contrast, our experiments with dogfish phagocytes have excluded the possibility that enzymes were taken up consequent to fusion of liposomes with the cell membrane, or after cell injury or death. The normal, post-phagocytic release of lysosomal β-glucuronidase (12, 20–23) was unaccompanied by leakage of cytoplasmic lactate dehydrogenase (20). Furthermore, ultrastructural cytochemistry demonstrated uptake of peroxidase-positive liposomes in the lysosomes of phagocytes, the acquisition of enzyme activity *only* by phagocytes, and no evidence of simple adherence of liposomes to, or fusion with, the cell surface. Finally, latency of HRP was demonstrated after uptake, and since glucose (ordinarily easily diffusible) was taken up as well, it is clear that for at least 30–60 min after engulfment, substantially intact liposomes (50% of HRP remained latent) were retained by the phagocytes. Experiments with free HRP, and with aggIgM.L[HRP, glucose] at 4°, but especially at 4° with cytochalasin, excluded the possibility that the bulk of apparent uptake was due to surface adsorption.

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