THE INTERACTION OF CATIONIC LIPOSOMES CONTAINING ENTRAPPED HORSERADISH PEROXIDASE WITH CELLS IN CULTURE

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ABSTRACT

Cationic liposomes composed of sphingomyelin, cholesterol, and stearylamine were prepared with horseradish peroxidase trapped inside. Stable particles were formed in which 10–12% of the enzymic activity appeared to be located at, or near, the outer surface of the liposome. Adsorption and uptake of liposomes by HeLa cells were followed cytochemically by electron microscopy and quantitated by enzyme assay and by the distribution and fate of particles labeled with [^{14}C]cholesterol and [^{125}I]horseradish peroxidase. The particles were adsorbed by HeLa cells at least 300 times as efficiently as was free horseradish peroxidase. Many of the particles remained at the cell surface, but numerous membrane-bound cytoplasmic inclusions were observed to contain peroxidase-staining material. In addition, many areas of the cell membrane gave a positive staining reaction. It was concluded that many particles (presumably the larger ones) did not gain access to the interior of the cells, many were phagocytized, and some enzyme was transferred to the cell membrane, perhaps as a result of fusion of the liposomal membrane with the cell membrane.

Lipid spherules, or liposomes, have been extensively studied since their initial description by Bangham et al. (1965). The particles may be prepared by the dispersion of dried films of phospholipids in an aqueous phase, and materials in the aqueous phase become trapped between the bilayer lamellae of the particles. Liposomes have proved to be useful models for membranes in permeability studies (Bangham et al., 1965), in their sensitivity to polyene antibiotics (Kinsky et al., 1968; Weissmann and Sessa, 1967) and susceptibility to immune lysis (Kinsky, 1972). Recently, some attention has been given to the possibility that particles of this type might be useful as carriers for various biological materials. Magee and Miller (1972) showed that cationic liposomes attached readily to cells in tissue culture, and that the cells were protected from virus infection by liposomes which contained antibody to the challenge virus. Several studies have appeared on the fate of enzyme-containing liposomes injected intravenously into experimental animals (Gregoriadis and Ryman, 1972 *a*, *b;* Gregoriadis and Buckland, 1973; Rahman et al., 1973).

The interaction of liposomes with cells might be presumed to fall into three categories: (*a*) the particles may attach by electrostatic (or other) interaction, but remain at the surface of the cells; (*b*) the attached particles might be phagocytized; or (*c*) the membrane system of the liposomes might fuse with that of the cells, in analogy to the interaction of parainfluenza viruses with cells (Dales, 1973). The present study examines the adsorption and uptake of liposomes by cells and the subsequent fate of an enzyme entrapped in the particles. Horseradish peroxidase was the enzyme chosen for study because a sensitive and reliable cytochemical assay is available (Graham and Karnovsky, 1966).

MATERIALS AND METHODS

Preparation of Liposomes Containing Horseradish Peroxidase

Sphingomyelin liposomes were prepared by dissolving 50 mg of sphingomyelin (highly purified, from bovine brain; Pierce Chemical Co., Rockford, Ill., or Sigma Chemical Co., St. Louis, Mo.), 10 mg cholesterol (Sigma Chemical Co., chromatographic standard grade), and 6 mg stearylamine (Chemicals Procurement Laboratories Inc., College Point, N. Y.) in 18 ml chloroform together with a small amount of methanol. The solution was divided among six 50-ml round-bottom flasks or three 100-ml round-bottom flasks, and the solvent was removed by flash evaporation on a rotary evaporator at room temperature. The flasks were flushed with N_2 and 0.3–0.5 ml of aqueous phase was added per flask. The aqueous phase contained 40 mg/ml horseradish peroxidase (grade II, Sigma Chemical Co., in 0.1 M phosphate buffer, pH 6.9). The films were dislodged from the glass by the use of a Vortex-Genie mixer (Scientific Industries, Inc., Springfield, Mass.). Usually two or three glass beads were added to aid in loosening the lipid film. The liposomes were removed from the flasks, and the flasks were rinsed with a small amount of aqueous phase. The liposomes were treated with ultrasonic vibration in intermittent 20-s intervals at 4–10°C for a total of 1–2 min with a Branson Biosonic III (Branson Instruments Co., Stamford, Conn.) equipped with a microprobe. The preparation was diluted with saline-phosphate (0.15 M NaCl and 0.01 M phosphate, pH 6.9) and the liposomes were collected by centrifugation at 60,000 *g* for 30 min in the 50 Ti rotor of a Beckman Ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pelleted liposomes were resuspended and washed twice more by centrifugation and stored at 0–5°C suspended in saline-phosphate.

Interaction of Liposomes with Cells

HeLa cells were grown on minimum essential medium (Eagle) containing 10% heat-inactivated calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 60-mm plastic petri dishes at 37°C in an atmosphere of 5% CO_2 in air. The monolayers were rinsed once with Hanks' balanced salts (HBSS), and 0.03–0.05 ml of liposomes in 1 ml of HBSS was added to each dish. Two to four dishes were used for each time interval, and each dish contained 2-3 × 10^6 cells. Liposomes were incubated with cells at 37°C for 30 min, and the unadsorbed liposomes then were removed with three washes of HBSS. Complete medium was added, and the dishes were returned to the 37°C incubator for the desired incubation period. The medium was decanted, and the cells on the monolayer were harvested by scraping with a rubber policeman into HBSS. Cells were recovered by a low-speed centrifugation in the cold at 200 *g* for 10 min. Any cells that had detached from the monolayer also were recovered from the incubation medium. The cells were washed once with 5 ml of HBSS and again recovered by centrifugation. The cell pellets were resuspended in 1 ml of HBSS. In some cases, the decanted medium and the scrape HBSS were further centrifuged at 60,000 *g* for 30 min to recover any free liposomes that might be present. All solutions were saved for determination of radioactivity and for enzyme assay.

Enzyme and radioactivity were released from the cells by the addition of 0.2 ml of a 5% solution of Triton X-100 to 1 ml of the resuspended cells followed by sonication for 1 min. A more complete release of the enzyme from intact liposomes was obtained by a further treatment of the extract with saponin (Kataoka et al., 1973). 0.2 ml of the extract was added to 2.5 ml H_2O followed by 65 μl saponin (100 mg/ml in 0.1 M Tris buffer, pH 7.5). The mixture was incubated 10 min at room temperature to lyse the particles; then 0.5 ml 10 N NaCl was added and the final volume was adjusted to 4.0 ml with water.

Aliquots were counted in Preblend 3a50 liquid scintillation cocktail (Research Products International Corp., Elk Grove Village, Ill.) on a Beckman liquid scintillation counter model LS-250.

Cytochemistry

HeLa cells were grown on cover slips (three per 60-mm plastic dish), incubated with liposomes, rinsed, and incubated in complete medium as above. At the time of harvest each cover slip was rinsed twice in Puck's balanced salts lacking calcium or magnesium before fixation *in situ* in 2.5% glutaraldehyde (Polysciences Inc., Warrington, Pa.) in 0.1 M cacodylate buffer (pH 7.2) for 10 min in an ice bath. The cover slips were rinsed several times in 0.1 M cacodylate, once in 0.1 M Tris buffer (pH 7.2) and stained with the diaminobenzidine reagent of Graham and Karnovsky (1966) (0.3 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in 0.1 M Tris buffer, pH 7.2, containing 0.01% H_2O_2) for 30 min at room temperature. The enzyme appeared to be completely stable to the glutaraldehyde fixation; no loss of either surface-reacting or internal peroxidase activity of liposomes could be detected with the quantitative colorimetric assay described below. Excess stain was removed by several rinses in Tris buffer, and the cells were fixed in

2% OsO_4 in 0.1 M cacodylate buffer for 30 min at 0–4°C. They were rinsed in water and dehydrated through a graded series of ethanols. The cells, still affixed to the cover slips, were embedded by inverting the cover slips over B.E.E.M. capsules (Better Equipment for Electron Microscopy, Bronx, N. Y.) filled with an Epon-Araldite mixture (Mollenhauer, 1964). The cover slips were removed by alternately heating and cooling the blocks after polymerization. Parallel thick (1–2 μm) and thin-sections were cut vertically and at various oblique angles to the original plane of attachment of the cells. The sections were observed and photographed with a Zeiss phase-contrast microscope and a Hitachi HU 11/E electron microscope.

Several different control incubations were included: (*a*) cells not previously exposed to liposomes were incubated in the complete staining reagent to check for the occurrence of endogenous peroxidase in HeLa cells; (*b*) cells previously exposed to liposomes were incubated in the staining reagent lacking H_2O_2 to confirm the peroxidative origin of the reaction product; (*c*) cells exposed to liposomes containing gamma globulin rather than horseradish peroxidase were incubated in the staining reagent as an additional check of the peroxidative origin of the reaction product and/or the induction of endogenous peroxidase by prior exposure to liposomes; (*d*) cells previously exposed to 1–150 μg/ml of horseradish peroxidase alone were incubated in the staining reagent to determine whether the free enzyme was adsorbed by HeLa cells.

Scanning Electron Microscopy

Cells grown on cover slips were fixed in glutaraldehyde as above, water rinsed, and freeze-dried (from water) in an Edwards freeze dryer (Edwards High Vacuum, Grand Island, N.Y.) at −70°C. Specimens were then mounted on aluminum stubs with a double stick plastic adhesive (3M Corp., St. Paul, Minn.) according to the technique of Vavra and Small (1969). A thin film of gold-palladium, approximately 10–30 nm in thickness, was evaporated onto the specimens in a vacuum of 5×10^{-6} torr while rotating on a "wobbler" stage. The specimens were then examined and photographed with a scanning electron microscope (Cambridge Mark IIa Stereoscan) at a 20 kV accelerating voltage.

Analysis

The enzymic activity of horseradish peroxidase was determined by a modification of the *o*-dianisidine method as described by Steinman and Cohn (1972 *a*). The reaction mixture contained 6 ml of 0.05 M phosphate buffer (pH 5.0), 0.06 ml of 0.03% (vol/vol) H_2O_2, and 0.5 ml of 1% (wt/vol) *o*-dianisidine (Sigma Chemical Co.) in methanol. The sample (0.01 ml) was mixed with 2.9 ml of reaction mixture and the rate of increase in optical density at 460 nm was determined at ambient temperature with a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Dilutions of the samples and standards were made in 0.1% bovine serum albumin in 0.05 M phosphate buffer, pH 5.0. A linear relationship between initial rate and nanograms of enzyme was obtained.

Protein was determined by the method of Lowry et al. (1951) with horseradish peroxidase as a standard. Protein in the liposomes was determined by the method of Kruski and Narayan (1973). From 0.1 to 0.2 ml of liposome suspension was spotted on 1.6 cm Whatman no. 1 filter paper disks and dried in a 60°C oven. Lipids were extracted from the disks by a 15-min immersion in 3 ml $CHCl_3$. The solvent was decanted and the disks were air-dried. Protein was extracted at room temperature for 30 min with 0.2 ml of 1 N NaOH, and quantitated by Lowry's method with standards processed in an identical manner.

Horseradish peroxidase was labeled with ^{125}I as follows (McConahey and Dixon, 1966): the enzyme (20 mg) was dissolved in 2.0 ml of 0.05 M phosphate buffer, pH 6.9, and chilled in an ice bath over a magnetic stirrer. 1 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, Mass.) was added followed by 20 μl of chloramine T (1 mg/ml). The reaction was allowed to proceed for 20 min and stopped by the injection of 20 μl of sodium metabisulfite (1 mg/ml). The reaction mixture was placed on a Biogel P-30 column (1 × 25 cm) and the iodinated protein eluted with saline-phosphate. 11% of the ^{125}I was incorporated into the protein.

RESULTS

Preparation of Liposomes Containing Horseradish Peroxidase

The amount of protein incorporated into liposomes was directly proportional to the amount of lipid employed to make the films and to the concentration of protein in the aqueous phase in the 10–40 mg/ml range. The addition of a divalent ion (Mg^{++}, 0.001 M) to the aqueous phase did not alter the amount of protein incorporated, nor did changing the pH in the range of pH 6 to pH 7. The usual preparation of sphingomyelin liposomes contained 66 mg mixed lipids (see Materials and Methods) and required the use of 3–4 ml of aqueous phase, containing 40 mg/ml of enzyme. The amount of protein incorporated into the lipid particles was 2.5–5% of that added in the aqueous phase (including that used for rinsing the flasks). Thus, typical liposome preparations contained 3–5 mg of protein, a maximum of 66 mg of lipid, and were suspended in a final volume of 3.0 ml. The amount of cholesterol incorporated into liposomes was measured by the use of [^{14}C]cholesterol and was 89–92% of that added.

Liposomes were stable for extended periods in the cold and were usually centrifuged and resuspended just before use. In several repeated tests, 10–30 μg/ml of horseradish peroxidase could be detected by enzyme assay in the supernate after 1–3 wk storage in the cold (Table I A). Several methods were examined for releasing enzymic activity in the preparation (Table I). Exposure to ultrasonic vibration in the presence of Triton X-100 liberated part of the activity for assay, and a further increase was obtained by treatment with saponin (Kataoka et al., 1973). Neither of these treatments affected the enzymic activity of free horseradish peroxidase. Although the data in Table I B make it appear that most of the activity was released, this was found not to be the case when the experiment was repeated with liposomes containing [^{125}I]horseradish peroxidase (Table I C). The colorimetric assay for protein in the particles was found to measure only 70–80% of the total present. From 20 to 30% of the radioactivity was extracted during the chloroform treatment of the liposome samples which had been dried on paper disks (see Materials and Methods). Radioactive horseradish peroxidase not enclosed in liposomes was quantitatively retained on the filter paper during this extraction. Experiments are being continued to determine if other methods may be employed to release enzyme more completely from the particles. However, it is possible that some enzyme inactivation occurs during preparation of the particles. Because reproducible enzyme release was obtained by the use of Triton X-100 followed by saponin, this method was used in subsequent experiments, and no corrections have been applied to the data.

Enzyme activity was readily detected on the surface of the liposomes by direct analysis of untreated liposomes (Table I). This suggested that some of the protein was either adsorbed to the exterior of the particles or trapped near the surface

TABLE I

Release of Enzyme Activity from Liposomes

	Protein		
	Enzyme assay	Lowry protein*	Corrected
		mg/ml	
(A) Original suspension (untreated)‡	0.14	0.89	(1.27)§
Supernate after centrifugation	0.03	0.06	0.06
(B) Resuspended liposomes (untreated)	0.12	0.84	(1.20)§
Triton X-100; ultrasonic, 4°C	0.42		
Triton X-100; ultrasonic, 37°C	0.68		
Saponin	0.88		
Saponin; ultrasonic, 4°C	0.96		
(C) Resuspended [^{125}I]liposomes (untreated)	0.11	1.22	1.74
Triton X-100; ultrasonic, 4°C	0.68		
Triton X-100; ultrasonic, 37°C	0.81		
Saponin	0.89		
Triton X-100 followed by:			
Saponin	0.97		
Saponin; ultrasonic, 4°C	0.92		

* The liposome samples were spotted on paper disks and extracted with chloroform, and protein was eluted with alkali for colorimetric assay (see Materials and Methods).

‡ The original suspension (A) of [^{14}C]cholesterol liposomes was assayed 1 wk after preparation. The liposomes were centrifuged at 60,000 *g* for 30 min and resuspended in their original volume (B). Aliquots were treated as indicated (see Materials and Methods) and suitable dilutions were assayed for total enzymic activity. In (C), liposomes containing [^{125}I]horseradish peroxidase were used. The preparation contained 1.84×10^5 cpm/ml and a calculated 1.81 mg/ml protein based upon the specific activity of the original [^{125}I]horseradish peroxidase.

§ Estimated from recoveries determined in part (C).

of the lipid bilayer during preparation. The latter explanation is favored because only small amounts of horseradish peroxidase were found to adsorb to liposomes after their preparation (Table II). The amount which could be adsorbed was the same whether the liposomes contained entrapped horseradish peroxidase or serum proteins.

Interaction of Liposomes with Cells

As reported previously (Magee and Miller, 1972), the inclusion of a cationic lipid in the liposomes was necessary to obtain significant adsorption to cells in tissue culture. The amount of horseradish peroxidase taken up by cells presented with the enzyme in the liposome particle was at least 300 times that obtained when the free enzyme was incubated with cells (Table III). In various experiments, 12–25% of the added liposomes became cell associated in 30 min (Table III). Since the amount adsorbed in 2 h did not greatly exceed this value, a time period of 30 min was selected for adsorption of the particles to the cells.

The specific activity (counts per minute per microgram enzyme) of the cell-associated material assayed uniformly higher than the original liposome preparation. [^{125}I]Liposomes contained 190 cpm/μg (uncorrected) while the cell-associated values were 240–280 cpm/μg calculated from the data in Table III. A similar calculation for the [^{14}C]cholesterol experiment showed 580 cpm/μg in

TABLE II

Adsorption of Horseradish Peroxidase to the Exterior of Liposomes

Composition of trapped aqueous phase	Horseradish peroxidase adsorbed		
	cpm	*Percent of added*	*μg/mg lipid*
Saline	280	0.15	2.1
Minimum media (Eagle) with 10% calf serum	370	0.19	2.8
Horseradish peroxidase (40 mg/ml)	465	0.24	3.6

Liposomes were prepared from 11 mg lipids with the indicated aqueous phase and then incubated in 0.4 ml 40 mg/ml [^{125}I]horseradish peroxidase (1.9×10^5 cpm), washed four times by centrifugation, and counted. The standard preparation of liposomes contained 50–60 μg peroxidase/mg lipid (total).

TABLE III

Uptake of Horseradish Peroxidase and Liposomes Containing Horseradish Peroxidase by HeLa Cells

Incubation conditions	Time	Average cell-associated	Enzyme
	min	%	*μg*
Horseradish peroxidase*			
Hanks' solution	30	<0.07	0.003
" "	60	<0.07	0.005
" "	120	<0.08	0.007
Complete medium	120	<0.03	0.009
Liposomes‡			
Hanks' solution	30	21.1	3.45
Hanks' solution (O°C)	30	22.6	2.55
Liposomes§			
Hanks' solution	30	14.2	3.53
Hanks' solution (O°C)	30	13.8	2.70

* 150 μg/ml of [^{125}I]horseradish peroxidase (7.5×10^5 cpm) was incubated in 2.0 ml of the indicated solution per petri dish containing approximately 2×10^6 cells.

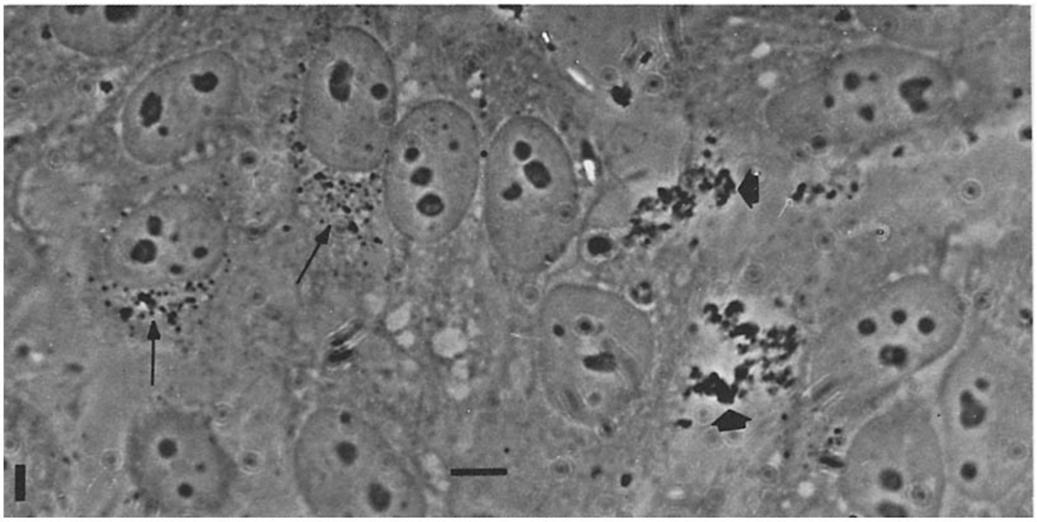
‡ 33 μl [^{14}C]cholesterol liposomes (1.84×10^4 cpm) in 1 ml Hanks' solution was added to each petri dish.

§ 33 μl liposomes containing [^{125}I]horseradish peroxidase (6.06×10^3 cpm) in 1 ml Hanks' solution was added to each petri dish.

FIGURE 1 Phase-contrast micrograph of a 1-μm thick section cut nearly parallel to the plane of the cell monolayer. The cells were briefly washed, fixed, and stained immediately after being incubated with liposomes for 30 min. Note the aggregates of liposomes at the surface of some of the cells (large arrows) as well as the numerous small, apparently individual liposomes in the cytoplasm of some of the cells (small arrows). Bar, 10 μm. × 725.

FIGURE 2 Scanning electron micrograph of control cells not exposed to liposomes. Note the absence of globular material, such as that seen in Fig. 3, on the cell surface. Bar, 10 μm. × 2,000.

FIGURE 3 Scanning electron micrograph of cells exposed to liposomes for 30 min before fixation. Note the presence of globular liposomes (arrows) on the surface of the cells. Bar, 10 μm. × 2,000.



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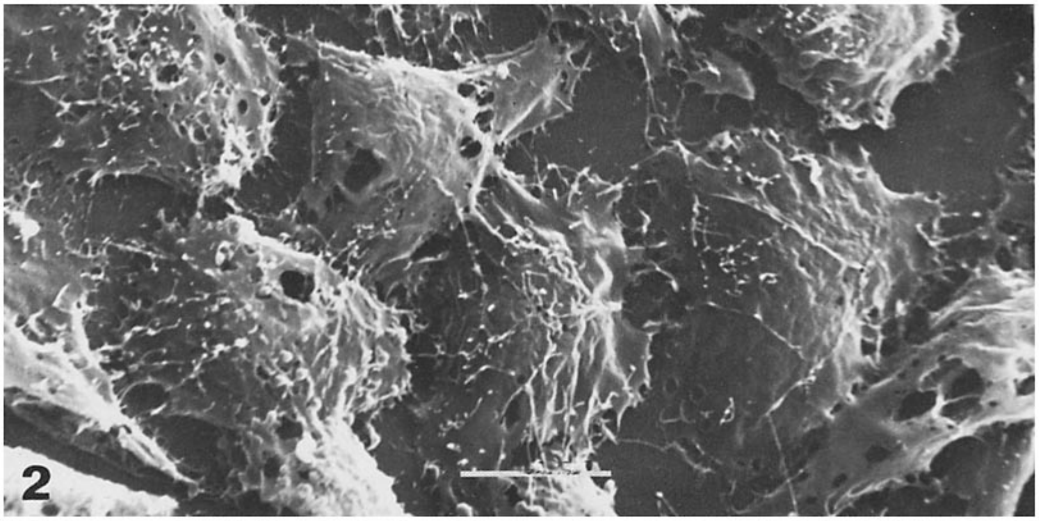

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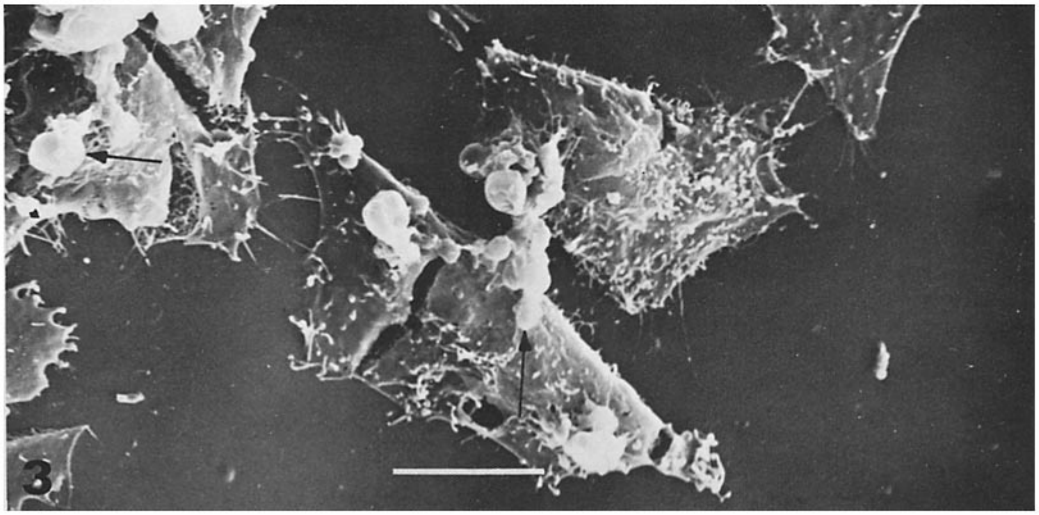

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the liposomes and 1,100–1,630 cpm/μg in the cell-associated material.

It would appear that the enzyme was less accessible for assay or suffered some loss in activity as a result of interaction with the cells. In the case of the [^{14}C]cholesterol particles, it also is possible that there was a preferential retention of the cholesterol by the cells or that there was some heterogeneity in the composition of the particles.

The initial attachment to cells may be chiefly electrostatic because the amount attached was not decreased by carrying out the adsorption at 0°C (Table III). Furthermore, when 0.25% trypsin or 0.1 M EDTA treatments were considered as alternatives to scraping as methods for harvesting cells, no decrease was noted in the amount of the added liposomes which remained cell associated after a 10-min incubation at 37°C that resulted in detachment of the cells from the surface of the dishes.

Incubation of cells with high levels of liposomes produced visible toxic effects. Thus, when cells were treated with 0.1–0.2 ml liposomes and examined after 24-h incubation in complete medium, from 20 to 25% of the cells were found free in the culture medium. Most of the detached cells remained intact as judged by their ability to exclude trypan blue, but many showed severe bulging and deformation of the cell membrane. All of the experiments reported were carried out with 0.03–0.05 ml of liposomes per petri dish where these effects were not detected.

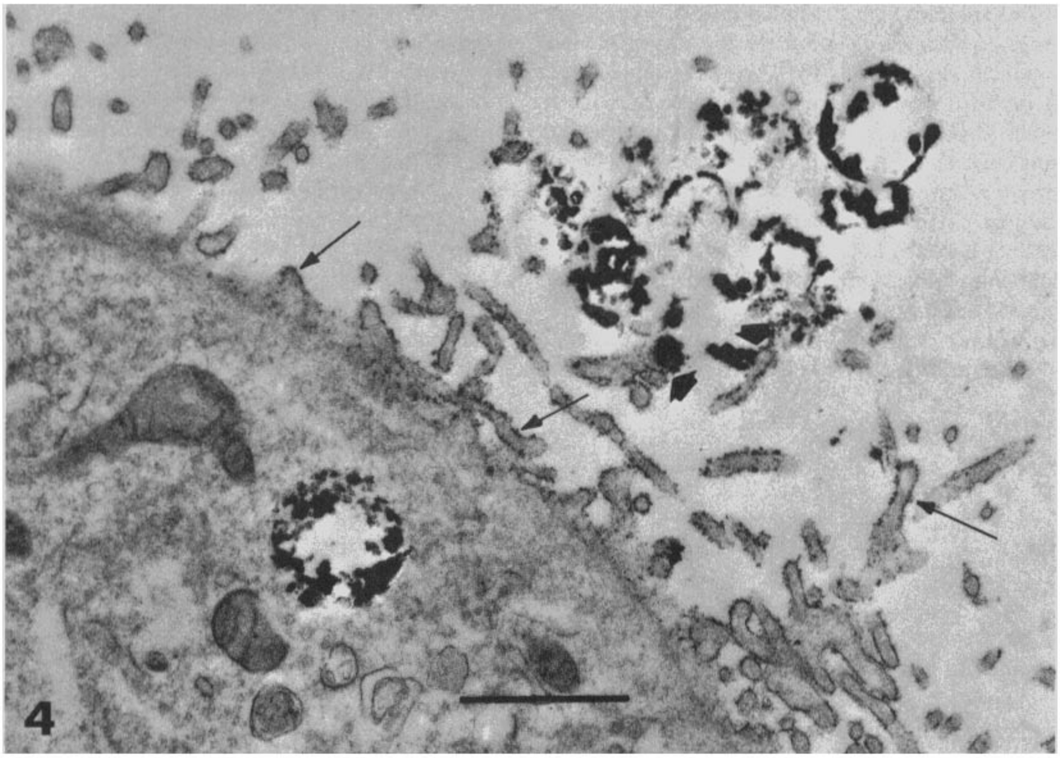
Observation of thick plastic sections (1–2 μm) of cells previously exposed to liposomes and subsequently stained by the diaminobenzidine procedure showed aggregates of peroxidase-positive material associated with the cell surface of many cells (Fig. 1). In addition, many cells appeared to contain numerous small peroxidase-positive particles within the cytoplasm (Fig. 1). The occurrence of single globular particles or aggregates of these particles in association with the cell surface was confirmed by scanning electron microscopy (Fig. 3). Such particles were absent from the surface of cells not previously exposed to liposomes (Fig. 2). Negative staining revealed the multilamellar substructure typical of the liposomes described by others (Kataoka et al., 1973). Measurement of scanning electron microscope images of liposomes attached to cells gave diameters of the particles generally varying between 1 and 2 μm, with an extreme size range of 0.8–5 μm.

Single particles or aggregates of stained particles also were observed by transmission electron microscopy in association with the surface of many cells (Fig. 4). The occurrence of pseudopodia in the vicinity of attached liposomes in many images suggests that these structures may be involved in the attachment of the liposomal material to cells. In addition, the outer surface of the cell membrane of both the cell proper and the pseudopodia stained positively for peroxidase in many areas (Figs. 4 and 5).

Particulate regions of peroxidase-positive material also were often observed within the cytoplasm of cells. Many of these cytoplasmic inclusions appeared to be membrane-bound (Fig. 5), indicating that the liposomes were taken up by phagocytosis. Micrographs of similar liposome-containing cells incubated in staining reagent lacking H_2O_2 contained aggregates of the presumed liposomal material, but did not contain the electron-dense reaction product (compare Figs. 5 and 6). The uptake of free horseradish peroxidase by the cells was insufficient to give a positive staining reaction even when cells were incubated with 150

FIGURE 4 Transmission electron micrograph of cells exposed to liposomes for 30 min and returned to complete medium for an additional 3.5 h before being fixed and stained with the diaminobenzidine reagent. Microvilli-like pseudopodia are associated with some of the extracellular liposomal material (large arrows). Reaction product, but apparently not liposomes, is also associated with the cell membrane of the microvilli-like extensions and of the cell proper (small arrows), and particulate, peroxidase-positive material is present in the cytoplasm. Not poststained. Bar, 1 μm. × 21,800.

FIGURE 5 Transmission electron micrograph of a cell exposed to liposomes for 30 min and returned to complete medium for an additional 3.5 h before being fixed and stained with the diaminobenzidine reagent. The peroxidase-positive liposomal material in the cytoplasm is in a membrane-enclosed vacuole (large arrows), presumably indicating that it has been phagocytized. Reaction product is also present on the cell membrane (small arrows). Not poststained. Bar, 1 μm. × 19,900.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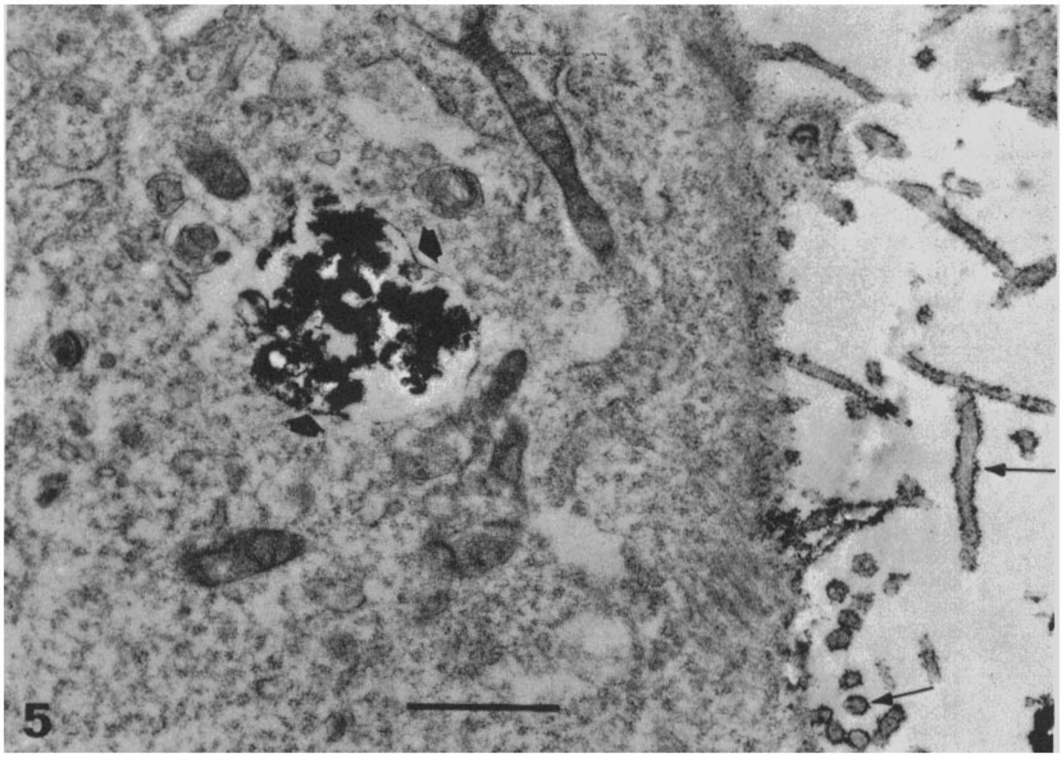
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μg/ml enzyme (Fig. 7). Similar lack of uptake was observed when 10 μg/ml of enzyme was incubated with cells continuously for 19 h. Detectable levels of product were not found in control cells incubated in the complete diaminobenzidine reagent (not illustrated), indicating that endogenous cellular peroxidases did not contribute to the observed staining patterns. Furthermore, reaction product was not detected in cells incubated with liposomes containing gamma globulin in place of horseradish peroxidase (Fig. 8), indicating that endogenous peroxidases were not induced in response to the interaction of the cells with liposomes.

Fate of Cell-Associated Liposomes

The general features just described for the interaction of liposomes with cells persisted for as long as 24 h. However, there appeared to be a decrease in both the size of the aggregates of liposomes associated with the cell surface and the size and staining granularity of the peroxidase-positive phagocytic vacuoles in the later samples as compared to earlier ones.

Quantitative studies were carried out to determine the distribution and fate of the components of the liposomes with time. Analysis of the metabolism of the particles was somewhat complicated because a portion of the liposomes did not appear to become firmly attached to the cells, or were broken down to smaller units which appeared in various subfractions during processing. This is illustrated in Table IV. In this case, cells were incubated for 1.5 h in complete medium after the initial adsorption period with [^{14}C]cholesterol liposomes. Note that over 2% of the radioactivity could be recovered from the incubation medium, and a similar amount was released during scraping of the cells to remove them from the petri dish. In this experiment, 24% of the dose was associated with the cell monolayer. Similar results were obtained by using liposomes which were labeled with [^{125}I]horseradish peroxidase, except that enzyme release more closely paralleled the release of radioactivity.

Fig. 9 shows the results of an experiment in which the amount of cell-associated material was determined over a 24-h period. The liposomes were labeled with [^{14}C]cholesterol. Increasing amounts of radioactivity appeared in the medium, and the amount of label associated with the cell monolayer declined to 86% of that observed immediately after adsorption. Enzyme activity recovered from the cells fell more rapidly to 65% of the initial by 24 h. The missing enzyme was recovered in the medium.

TABLE IV

Distribution of Liposome Components in Medium and Cells during a 2-h Incubation

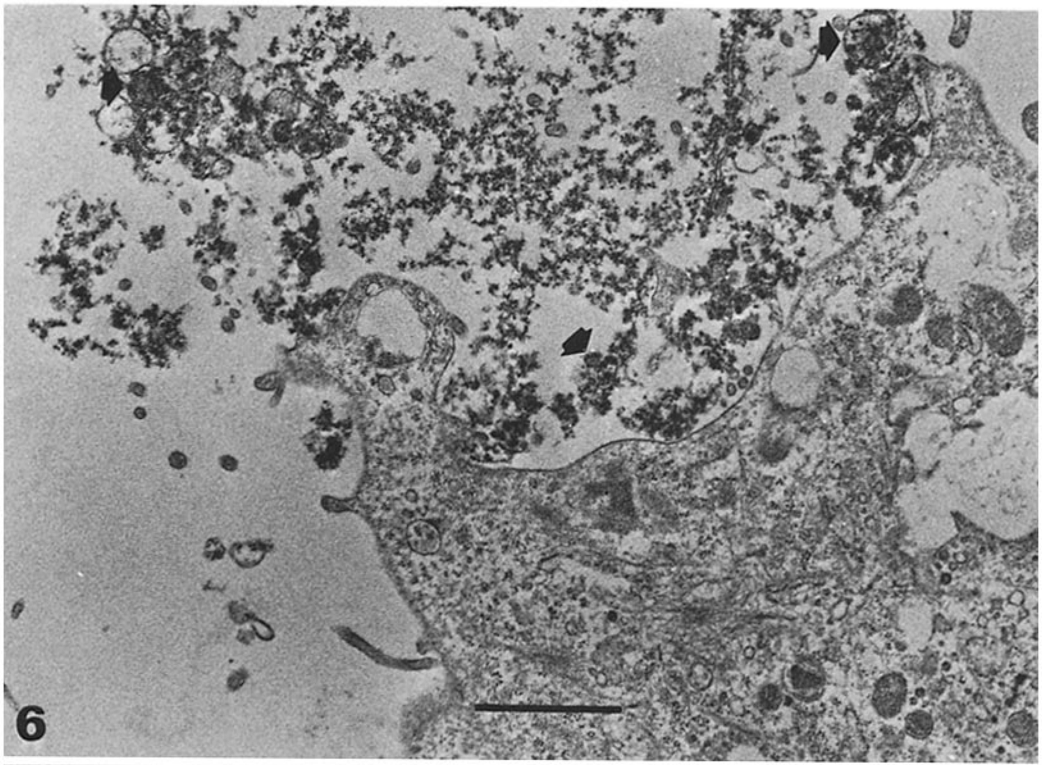
Fraction	Radioactivity		Enzyme
	cpm	*percent dose*	*μg*
Incubation medium			
Low-speed pellet	698	1.27	0.24
Low-speed supernate	420	0.76	0.85
Harvest medium and wash	1,250	2.29	0.44
Cells	13,300	24.1	8.80

Cells in three petri dishes were exposed to [^{14}C]cholesterol-containing liposomes for 30 min, washed, and incubated in growth medium for an additional 1.5 h before harvest (see Materials and Methods).

FIGURE 6 Transmission electron micrograph of cells exposed to liposomes for 30 min and returned to complete medium for an additional 3.5 h before being fixed and stained in diaminobenzidine reagent lacking H_2O_2. Note the presence of liposomal material (arrows) but the absence of electron-dense reaction product indicative of peroxidase activity. Not poststained. Bar, 1 μm. × 19,300.

FIGURE 7 Transmission electron micrograph of a portion of a cell exposed to 150 μg/ml of free horseradish peroxidase for 30 min and returned to complete medium for 2.5 h before fixation and staining with the diaminobenzidine reagent. Peroxidase activity was not detected either at the membrane or inside the cells. Not poststained. Bar, 1 μm. × 22,700.

FIGURE 8 Transmission electron micrograph of a portion of a cell exposed to liposomes prepared with gamma globulin rather than horseradish peroxidase. Exposure was carried out for 30 min followed by 2.5 h in complete medium before fixation and staining with the diaminobenzidine reagent. Arrows indicate the peroxidase-negative liposomes. Not poststained. Bar, 1 μm. × 33,800.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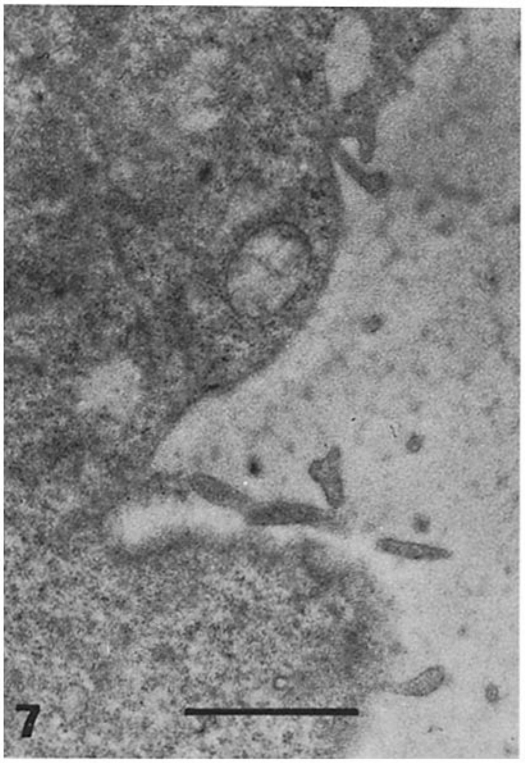
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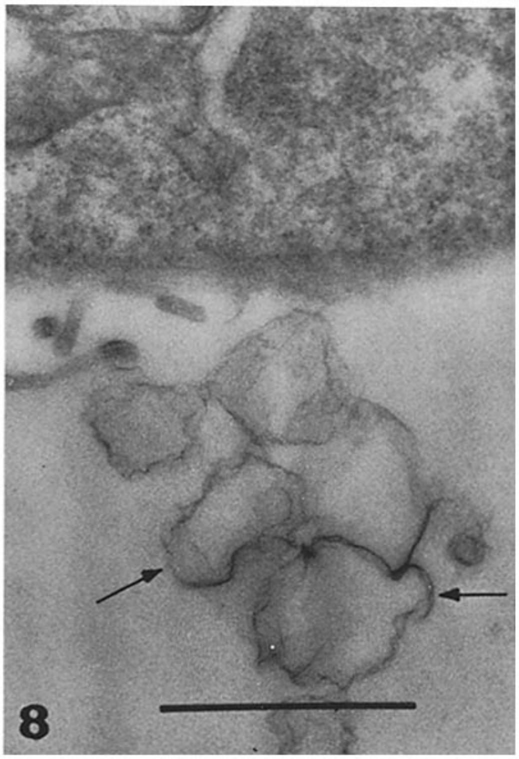
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Thus, cholesterol seemed to be preferentially retained by the cells. A similar experiment was carried out with liposomes containing [^{125}I]horseradish peroxidase (Fig. 10). By 28 h, radioactivity and enzymic activity associated with the cell monolayer had declined to one-third of initial values. The cell-associated specific activity remained constant. Cells and debris released into the medium were collected separately by low-speed centrifugation and analyzed for radioactivity and enzyme activity. Increasing amounts were recovered in this fraction at the later time periods, indicating possible toxic effects to the cells. Aggregates of liposomes released from cells also would be expected to be found in this fraction. In both of these experiments, total recovery of radioactivity and enzyme activity did not decline with time, indicating that only minimal enzymic digestion of the protein by the cells had occurred.

In preliminary experiments, cells were treated with liposomes and then subcultured three times over a period of 1 wk to determine the persistence of enzymic activity. Over 90% of the activity was lost into the medium during the first passage of the cells. The remaining enzyme disappeared from cells with a half-life of 12–16 h.

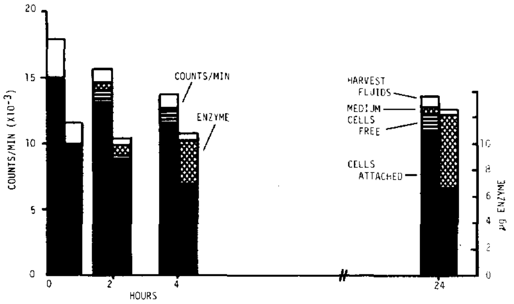

FIGURE 9 The fate of [^{14}C]cholesterol and peroxidase activity in cells exposed to liposomes for 30 min and returned to complete medium for various lengths of time. "Cells free" refers to material recovered by low-speed centrifugation from the incubation medium at the time of harvest (see Table IV). The figures are totals for three petri dishes.

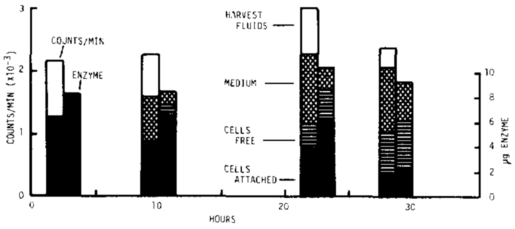

FIGURE 10 The fate of [^{125}I]horseradish peroxidase and peroxidase activity in cells exposed to liposomes. Conditions as in Fig. 9.

DISCUSSION

Liposomes were prepared which appeared to be stable and which contained 5–6% protein. It is interesting that surface enzymic activity could be detected that was about 10–12% of the total present. Sessa and Weissmann (1970) detected a similar amount of activity in intact particles containing lysozyme, and we have reported that antibody was detectable at or near the surface of particles containing antiviral human IgG (Magee and Miller, 1972). The exact location of the surface-reacting material cannot be determined from the present experiments, but calculations from the data in Table II show that no more than a small fraction of the surface activity could result from physical adsorption to the outer surface of the particles. It seems possible that some protein may become embedded in the lipid bilayer during sonic treatment of the particles, particularly if the molecule contains appreciable hydrophobic regions.

The particles often appeared to be attached to cells as aggregates. It should be pointed out that the present method of processing and embedding for electron microscopy may not accurately preserve the ultrastructure of the liposomal material. The dehydration and embedding procedures were found to extract [^{14}C]cholesterol almost quantitatively from the particles (unpublished observations). Studies are in progress to overcome this difficulty.

The cationic liposomes adsorbed very readily to cells, and at least 300 times as much horseradish peroxidase became cell associated as could be obtained by using the free enzyme. Thus, from Table III, when 2×10^6 cells were incubated with 150 µg/ml enzyme, less than 0.01 µg became cell associated, while incubation with liposomes (containing 33 µg enzyme/ml) resulted in 2.5–3.5 µg being retained by the cells (uncorrected values). The latter figure was a nonsaturating amount, and the limiting factor for liposome attachment appeared to be the amount of available surface area of the cell. Initial interaction of the particles with cells seemed to rely only on the charge attraction between the positively charged particles and the negatively charged cells and was not altered by temperatures between 0 and 37°C.

A comparison might be made between this study and those reported by Steinman and Cohn (1972 *a,b*). These authors compared the uptake of free peroxidase and that of aggregated peroxidase-antiperoxidase by peritoneal macrophages. Free peroxidase was taken up to a very minor degree with only small amounts detectable in lysosomal vesicles. Their results showed that no more than traces of the enzyme were retained at the surface of the cells, although other workers have reported that some cells show positive surface staining (Cotran and Litt, 1970). In contrast, antibody-peroxidase aggregates were taken up a rate 4,000 times that of the soluble enzyme and were present on the cell surface and located in phagosomes. Enzyme activity was degraded by the cells with a half-life of 14–18 h. Brandes et al. (1972) also have observed uptake of free horseradish peroxidase on the surface and in small vesicles in WI-38 cells in culture. We were unable to detect a positive stain either on the membrane or in vesicles after incubating HeLa cells with free enzyme for various lengths of time.

HeLa cells appear to have considerably less ability to phagocytize particulate materials than do peritoneal macrophages. This may explain the continuing presence of many liposomes at the surface of cells. Other contributing factors undoubtedly were the relatively large size of most of the liposomes in these preparations and their tendency to aggregate. These same factors may contribute to the toxicity of the preparations, particularly where clumped particles attached to individual cells. The only intracellular peroxidase detected was present in typical phagocytic vesicles, where lysosomal digestion should occur. It seemed puzzling that no decrease in total enzymic activity was observed over a 24-h period. However, if only 10–15% of the attached liposomes were engulfed and found in lysosomes, the anticipated drop in enzymic activity would not be detected. A continuing loss of activity was observed after a subculture of the treated cells, giving a half-life of 12–16 h.

An interesting feature of these experiments was the rather frequent observation that peroxidase-staining material was associated with the cell membrane and particularly with the pseudopodia. This suggests either that some of the particles fused with the cell membrane or that the enzyme became associated with the cell surface in some other fashion. In any case, if the location of the reaction product accurately reflects the location of the enzyme, then the enzyme became bound either to the liposomal membrane (as discussed above) or the cell membrane rather than being trapped exclusively in an aqueous phase between membranes. That the surface staining was acquired via the fusion of peroxidase-laden liposomal membranes with the cell membrane is indicated by the failure of free peroxidase to bind to HeLa cells in quantities detectable by the cytochemical stain.

An ideal delivery system utilizing liposomes might be visualized as one in which the membrane of the particle would fuse with the membrane of the target cell, simultaneously discharging the contents of the liposomes directly into the cytoplasm. Such a possibility seems quite feasible by the proper manipulation of the lipid composition of the particle. Several recent reports bear on this question. Grant and McConnell (1973) have shown that liposomes may fuse with the cell membrane of *Acholeplasma laidlawii*, and Papahadjopoulos et al. (1973) showed that liposomes composed of a wide range of phospholipids can be used to induce fusion of mammalian cells in tissue culture. Only neutral or negatively charged particles were used, and more rigid, cholesterol-containing particles were less effective for inducing cell fusion. Gregoriadis and Buckland (1973) gave evidence that liposomes containing invertase could alleviate a model storage disease in tissue culture.

Several studies of liposomes for in vivo applications already have appeared which may be presumed to rely upon intracellular uptake of liposomes. Gregoriadis and Ryman (1972 *a,b*) have shown that enzyme-containing liposomes were rapidly removed from the circulation in the rat, and were trapped mainly in the liver, with lesser amounts appearing in the spleen and lungs. Cell fractionation studies showed liposome components to be in the lysosomal fraction of liver cells. An interesting report showed that liposomes containing an entrapped chelating agent strongly promoted clearance of plutonium from livers of mice when animals had been previously dosed with high levels of the heavy metal (Rahman et al., 1973). Recently, we have shown that liposomes may be prepared containing entrapped polyinosinic: polycytidylic acids, and that the particles greatly potentiate the interferon response in mice over that obtained with the free double-stranded polynucleotide (S. X. Straub, R. F. Garry, and W. E.

Magee. 1974. *Infect. Immun.* In press).

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