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COVALENT ATTACHMENT OF HORSERADISH PEROXIDASE TO THE OUTER SURFACE OF LIPOSOMES

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Summary

We describe a method by which horseradish peroxidase may be attached covalently to the surface of liposomes under conditions which permit minimal non-covalent association of the enzyme with the lipids. The coupling method adopted does not allow the formation of homopolymers of liposomes or peroxidase. For phosphatidylethanolamine/phosphatidylcholine and stearylamine/phosphatidylcholine vesicles, minimal disruption of vesicular structure is observed, whilst for phosphatidylserine vesicles, the lipid-protein complex appears to form structures much smaller than 25 nm in diameter. Stearylamine/phosphatidylcholine vesicles have been shown to retain entrapped inulin, and activity measurements for the peroxidase suggest that it is located exclusively on the external surface of the liposome membrane. Peroxidase can be localized histochemically which has permitted the morphological study of the coated liposomes and their interactions with cells.

Introduction

The ability of liposomes to encapsulate water-soluble compounds within their internal aqueous spaces has led to the proposal that they be used as depots for the delivery of drug in vivo [1]. The additional possibility is also that target specificity might be conferred on liposomes by attaching specific receptors to their surfaces [2]. The manner in which liposomes interact with cells is presently uncertain [3] and may be of prime importance in their use as

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drug carriers. Fusion of liposomes with the cell surface should result in the intracytoplasmic injection of the liposome contents and the incorporation of the liposome membrane into the plasma membrane. Endocytosis of liposomes would result in their intracellular delivery, but might expose them and their aqueous contents to the lysosomal system of the cell. Simple adhesion of liposomes to the cell surface could only produce intracellular delivery of their contents by passive diffusion.

In order to distinguish between these possibilities, morphological and biochemical markers are required, the fate of which will reliably reflect the overall fate of the liposome membrane components. With these points in mind we have investigated the feasibility of attaching proteins to the external liposome surface via a covalent bond to a phospholipid head group. A priori, the method of conjugation used must be efficient in aqueous solution under mild conditions, and should degrade neither the lipids nor the protein. The formation of homopolymers of either liposomes or proteins is also undesirable, since this will reduce the yield of the desired products and will produce a mixture of products that may prove difficult to separate. It must be possible to establish conditions for covalent attachment in which liposome-protein complexes are not formed by non-covalent attachment as otherwise it will be difficult to asses the extent of the covalent interaction between the liposomes and the protein.

In order to measure the extent of coating of the external surface of the liposomes, preparations of liposomes with reproducible, and preferably known, surface area : phospholipid ratios should be used, and an ideal model protein should have properties that will permit proof of its exclusive location on the external surface of the liposomes. We have approached these problems by investigating the covalent attachment of horseradish peroxidase to liposomes using the method of Nakane and Kawaoi [4]. Peroxidase exhibits very low non-specific binding to liposomes under the conditions used for coupling, and the coupling method of Nakane and Kawaoi only permits binding of peroxidase to liposomes, without formation of homopolymers. Small unilamellar vesicles and the potentially more useful large unilamellar vesicles have been used for this study, and multilamellar vesicles, which might be expected to exhibit very low and irreproducible surface area : phospholipid ratios, have been avoided. By using an enzyme as a model protein, it is possible to demonstrate its presence on the external surface of the liposomes by its availability for interaction with its substrate. Because peroxidase can be localized using electron microscopy, the interaction of the liposome-enzyme complex with the cell surface can be visualized. Our observations have been reported in preliminary form elsewhere [5].

Materials and Methods

Reagents. Phosphatidylethanolamine and phosphatidylcholine from egg yolks and phosphatidylserine from bovine brain were obtained from Lipid Products (Epsom), and stearylamine was obtained from K and K Laboratories (Plainview). Inulin, type VI horseradish peroxidase and bacitracin were obtained from Sigma (London). 2,2′-Azino-di-(3-ethylbenzthiazolinesulphonate) [6], was obtained from Boehringer-Mannheim and Sephadex G-50 and Sepharose 6B from Pharmacia (Uxbridge). [^{3}H]Inulin was obtained from

The Radiochemical Centre, Amersham. All other chemicals were reagent grade or better.

Preparation of liposomes. Small unilamellar vesicles were prepared from mixtures of phosphatidylethanolamine and phosphatidylcholine, or from phosphatidylserine alone, by sonication of lipid (approx. 40 μmol) in 3 ml of 10 mM sodium succinate, pH 6.0, with continuous slow nitrogen purging for 1—1½ h in a Baird and Tatlock 50 W ultrasonic cleaning bath at 15°C. After sonication the suspension was centrifuged at 400 000 × *g* for 1 h to remove the larger liposomes. The supernatant was carefully removed and stored under N_2 at 4°C, and was analyzed for its phosphorus content prior to use. Peroxidation of the lipid was negligible under these conditions. Electron-microscopic observation of negatively stained phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles revealed a homogeneous population with diameters between 25 and 50 nm (Fig. 1).

Reverse phase solvent evaporation vesicles were prepared from mixtures of phosphatidylcholine and stearylamine by the method of Szoka and Papahadjopoulos [6]. 1 ml succinate buffer, pH 6.0, either alone or containing inulin or peroxidase, was added to 3 ml diethyl ether containing approx. 40 μmol phospholipid. The mixture was sonicated for 2 min under N_2 and the ether was removed on a rotary evaporator. 70—90% of the phosphorus in the resultant aqueous suspension was found to sediment when centrifuged at 400 000 × *g*

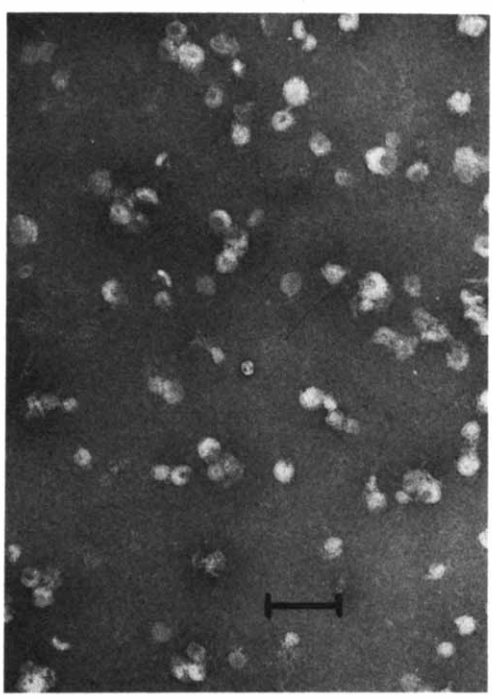


Fig. 1. 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles negatively stained (bar = 0.1 μm).

for 1 h. Non-entrapped solute was separated from the solvent evaporation vesicles by four successive sedimentations of the vesicles.

Activation and coupling of peroxidase. Peroxidase was activated for attachment to liposomes by a modification of the method of Nakane and Kawaoi [4]. 10 mg of peroxidase in 1 ml of 1 M $NaHCO_3$ (pH 8.1) were treated with 50 μl fluorodinitrobenzene (1%, w/v) in ethanol for 1 h at room temperature with gentle mixing. 1 ml $NaIO_4$ (0.06 M) was then added and the solution was mixed in the dark for 30 min. 1 ml ethanediol (0.32 M) was added to neutralize the residual $NaIO_4$ and the mixture was left for 1 h in the dark. $NaIO_4$ treatment changed the colour of the peroxidase from brown to green, but ethanediol treatment reversed the change. Activated peroxidase was separated from the excess reagents by desalting on Sephadex G-50 into 10 mM succinate buffer. In some experiments the conditions for fluorodinitrobenzene blocking were varied. For coupling to phosphatidylcholine/stearylamine solvent evaporation vesicles the fluorodinitrobenzene blocking was omitted, and for coupling to phosphatidylserine small unilamellar vesicles fluorodinitrobenzene blocking of peroxidase was performed at pH 9.5 in 1 M carbonate buffer.

For coupling to liposomes, the activated peroxidase (10 mg) in 5 ml buffer was mixed with 1 ml of liposome suspension (approx. 4 μmol phosphorus) and 0.5 ml of 1 M carbonate buffer (pH 9.5) was then added to raise the pH to 9.5 for reaction. After 2 h, 2 mg $NaBH_4$ were added and the solution left at room temperature overnight. The mixture was neutralized to pH 6.0 with HCl and the liposomes and non-bound enzyme were separated. Solvent evaporation vesicles were separated by sedimentation at 400 000 × *g* for 1 h, and were resuspended and washed four times. Small unilamellar vesicles were concentrated to 1.5 ml on an Amicon PM10 membrane at 3—4 lb/inch^2 and applied to a 50 × 1.5 cm Sepharose 6B column eluted with 10 mM succinate buffer.

Analysis. Phosphorus analysis was performed by using the method of Bartlett [7] with modification. Approx. 0.1 μmol phosphorus in not more than 200 μl buffer was heated to 215°C with 50 μl of 72% $HClO_4$ for 20 min. After cooling, the sample was mixed with 4.6 ml of 0.22% $(NH_4)_2MbO_4$ in 0.55 M H_2SO_4, and 0.2 ml Fiske-Subarrow reagent. The mixture was heated to 100°C for 7—10 min, cooled, and the absorbance measured at 660 nm. Nitrogen content was measured by using the method of Jaenicke [8] using the digestion method of Sloane-Stanley [9] without Teflon 'rodlets'. 50 μl $HClO_4$ were used for a 1 h digest on a regulated 'King pattern' digestion stand, and the reagent volumes used by Jaenicke for the colorimetric measurement of NH_3 were doubled. The procedure of Jaenicke for ashing was tried, but proved ineffectual for digesting phosphatidylcholine, even when extended to 1 h. Determination on exhaustively dried samples revealed the nitrogen content of the peroxidase used to be 11.4%. The nitrogen : phosphorus ratio for phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine was constant for samples up to 0.2 μmol and was found to be 0.93. Determination of the protein : phospholipid ratios were based upon these two observations.

Peroxidase activity. Peroxidase activity was measured using the dye, 2,2'-azino-di(3-ethylbenzthiazolinesulphonate) [6]. 2.2 ml of 0.1 M $NaHPO_4$ buffer (pH 7.0), 0.7 ml of 55 mg/ml 2,2'-azino-di(3-ethylbenzthiazolinesulphonate) [6], and 0.03 ml enzyme were mixed in a 3 ml cuvette and equilibrated at

25°C. The reaction was started by addition of 0.1 ml of 0.89 mM H_2O_2 and was monitored at 436 nm on a Unicam SP800 recording spectrophotometer. The activity was calculated as follows:

$$\text{Unit/ml peroxidase} = \frac{\text{absorbance/min} \times 3.03}{0.03 \times 29.3}$$

To ascertain the latency of liposome-associated peroxidase, 20 μl of 10% Triton X-100 were added to the cuvette to disrupt the liposomes after the activity for the intact vesicles had been established. This quantity of Triton X-100 is sufficient to disrupt the vesicles and larger amounts of Triton X-100 were found to inhibit enzyme activity.

Electron microscopy

Liposomes. Liposomes were negatively stained using 1% bacitracin as a wetting agent and 2% ammonium molybdate stain according to the method of Gregory and Pirie [10]. For sectioning, liposomes were suspended in molten 1% agarose which was allowed to solidify and diced into 1 mm cylinders, which were then fixed in a mixture of glutaraldehyde and OsO_4, stained with uranyl acetate, dehydrated and embedded [11]. To visualize peroxidase activity the agar cylinders were incubated in a 3 mg/ml solution of diaminobenzidine for 30 min prior to the addition of 100 vol. H_2O_2 to a final concentration of 0.01%. After 5 min, the cylinders were washed thoroughly, and fixed and embedded as described above.

Cells. Approx. 2 nmol of peroxidase-coated phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles or phosphatidylcholine/stearylamine solvent evaporation vesicles were injected intraperitoneally into mice in 1 ml phosphate-buffered saline. After various times the mice were killed and the peritoneum washed out with 2-ml phosphate-buffered saline containing 1% bovine serum albumin. This exudate was fixed in 1% glutaraldehyde in phosphate-buffered saline for 5 min, washed, then incubated in diaminobenzidine for 5 min. The cells were fast-fixed in OsO_4 and processed for electron microscopy.

Isoelectric focusing. Isoelectric focusing was performed in LKB pH 3.5—9.5 Multiphor PAG plates run at not more than 30 W for 2 h at 10°C. The pH gradient was determined by removing a 5 mm wide strip and cutting it into 10 mm sections, which were soaked overnight in 1 ml portions of degassed distilled water. The pH of the solutions were taken as the values at intervals along the gel. Gels were stained in 0.05% Coomassie blue, 0.1% $CuSO_4$, 10% acetic acid, and 25% ethanol in water for 4 h, semi-stained overnight in the above solution with only 0.01% Coomassie blue, and destained in 0.1% $CuSO_4$, 10% ethanol and 10% acetic acid in water for as long as necessary [12].

Results

Isoelectric focusing. Fig. 2 shows the isoelectric focusing pattern for unmodified and fluorodinitrobenzene-blocked peroxidase. This preparation of peroxidase (Sigma type VI) when unmodified exhibits a diffuse major band with an isoelectric point between pH 7.5 and 8.0, and a minor band at pH 6.5. Reac-

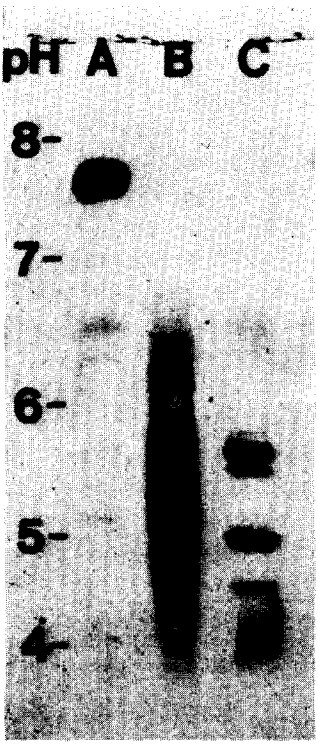


Fig. 2. Isoelectric focusing of peroxidase. A, unmodified peroxidase; B, fluorodinitrobenzene-treated at pH 8.1; C, fluorodinitrobenzene-treated at pH 9.5.

tion with fluorodinitrobenzene modifies the lysine residues and shifts the isoelectric points of the components to a lower pH. Reaction with fluorodinitrobenzene at pH 8.1 as specified by Nakane and Kawaoi [4] produces a heterogeneous product the components of which have isoelectric points between pH 3.8 and 6.8. This product is said [4] to have dinitrophenyl groups on two of its six lysine residues. Reaction with fluorodinitrobenzene at pH 9.5 appears to have a greater effect upon the isoelectric points of the peroxidase since the product contains major bands at pH 5.9 and 5.2, minor bands at pH 4.5 and 5.8 and diffuse minor bands at pH 6.8 and 4.0. No other treatment used to activate peroxidase for coupling has any effect upon its isoelectric focusing pattern.

Where peroxidase was to be linked to liposomes which bear a net surface charge, this information was used to select both the conditions for fluorodinitrobenzene blocking and the pH for separation of liposomes from unbound enzyme. For coupling to phosphatidylserine vesicles, peroxidase reacted with fluorodinitrobenzene at pH 9.5 was used, since above pH 6.8 all of the isoenzymes so treated should be net negatively charged. By coupling the liposomes and enzyme at pH 9.5 and separating the products at pH 8.0, both enzyme and liposomes should always be negatively charged and nonspecific binding due to electrostatic interactions should be minimized. For peroxidase coating of

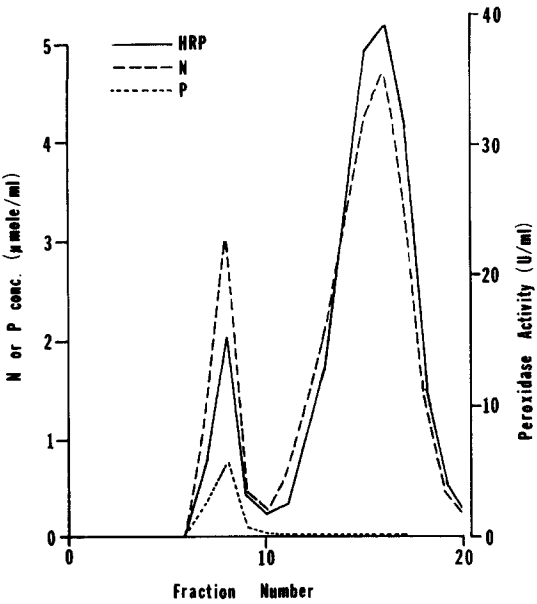


Fig. 3. Elution profile of peroxidase coupled to 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles. 4 μmol of lipid were reacted with 10 mg enzyme and chromatographed on a 1.5 × 45 cm Sepharose 6B column. 3.8-ml fractions were collected.

stearylamine liposomes fluorodinitrobenzene blocking was omitted. Although electrostatic interaction might occur at pH 9.5, at pH 6, which was used for separating the products, the enzyme is positively charged and should dissociate from the liposomes unless it is covalently linked to their surface.

Binding to phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles. Fig. 3 shows the elution profile on Sepharose 6B for 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles and peroxidase which have been coupled. The profile shows two peaks, both containing enzyme activity and nitrogen, but only one containing phosphorus. The phosphorus-containing peak is eluted in the same volume as unmodified small unilamellar vesicles (not shown) and therefore, no reduction in the size of the vesicles appears to occur when coupled to peroxidase. Since small unilamellar vesicles are excluded by Sepharose 6B, no increase in vesicle size would be detected. The absence of phosphorus in the unbound peroxidase peak confirms that the phosphatidylethanolamine-peroxidase conjugate remains a part of the liposome and does not dissociate to give monomeric peroxidase-phosphatidylethanolamine.

Table I shows that the nonspecific binding of peroxidase to neutral liposomes is very low. No peroxidase activity is detectable in the liposome fractions, if unmodified peroxidase is mixed with 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles and subjected to the conditions of coupling. The presence of phosphatidylethanolamine is essential for the binding of peroxidase to phosphatidylcholine/phosphatidylethanolamine small

TABLE I

THE BINDING OF PEROXIDASE TO VESICLES UNDER VARIOUS CONDITIONS

4 μmol lipid were reacted with the amount of peroxidase shown. Final protein:lipid ratios were determined from the nitrogen and phosphorus content of the vesicles after purification on Sepharose 6B. Means are given ± S.D. Lipid compositions are expressed as molar ratios. PC, phosphatidylcholine; PE phosphatidylethanolamine.

Number of experiments	Lipid composition of vesicles	Initial amount of protein (mg)	Final protein:lipid ratio (μg protein/μmol phospholipid)
Controls			
1	2:1 PC/PE	10 *	0 ***
1	PC	10	0.5 ***
1	2:1 PC/PE	10 **	21 ± 7
Coupled			
3	2:1 PC/PE	10	250 ± 50
1	2:1 PC/PE	8.33	210
1	2:1 PC/PE	16.67	216
1	2:1 PC/PE	25.00	172
3	1:2 PC/PE	10	230 ± 70

* Peroxidase was not activated.

** Peroxidase was activated and reduced.

*** Peroxidase estimated by enzyme activity.

unilamellar vesicles, since if coupling is performed in the normal way but with phosphatidylcholine small unilamellar vesicles very little peroxidase activity is coupled. The presence of aldehydes on the peroxidase molecule is essential for binding since very little binding occurs if the activated peroxidase is reduced with $NaBH_4$ prior to coupling.

Table I also shows the amount of peroxidase which can be bound to phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles under various conditions. Coupling of peroxidase to 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles appears to be maximal when 3 μmol phospholipid are reacted with 8 mg enzyme, since a 2- or 3-fold increase in the amount of enzyme produces no discernible increase in the amount of enzyme bound to the vesicles. This degree of binding may constitute a complete coating of the external surface of the vesicles since increasing the mole fraction of phosphatidylethanolamine from 0.33 to 0.66 has no discernible effect upon the amount of peroxidase bound to the vesicles.

Binding to phosphatidylserine small unilamellar vesicles. The results of peroxidase coupling to small unilamellar vesicles composed of phosphatidylcholine and phosphatidylethanolamine are sharply contrasted by our findings with phosphatidylserine small unilamellar vesicles, depicted in Fig. 4. Phosphatidylserine small unilamellar vesicles appear from chromatography (Fig. 4b) and from electron microscopic observation to contain a proportion of particles smaller than 25 nm in diameter, since the elution profile shows a shoulder on the trailing side of the liposome peak. Peroxidase, when treated with $NaBH_4$ after fluorodinitrobenzene treatment at pH 9.5 and $NaIO_4$ oxidation, appears to elute as a single peak (Fig. 4a). When phosphatidylserine small unilamellar

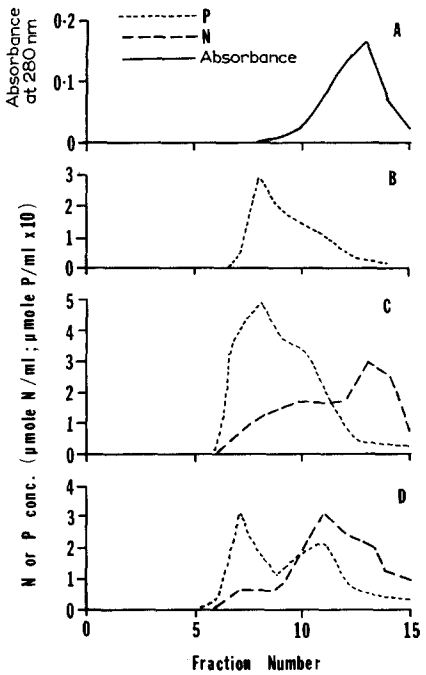


Fig. 4. Elution profile of peroxidase and phosphatidylserine small unilamellar vesicles. A, modified and reduced peroxidase alone; B, vesicles alone; C, vesicles plus peroxidase, mixed without coupling; D, peroxidase and vesicles coupled. A single 1.5 X 45 cm Sepharose 6B column was used for these experiments. 3.8-ml fractions were collected.

vesicles and such treated peroxidase are mixed at pH 8.5 and separated on Sepharose 6B (Fig. 4 c) the shoulder on the trailing side of the liposome peak is more pronounced and the peroxidase peak has a shoulder on its leading edge, which elutes in the same regions as the low molecular weight phospholipid particles. This shifting of enzyme and phospholipid to an intermediate elution volume between the two individual components is more apparent when the peroxidase and the liposomes are coupled (Fig. 4d). The phosphorus now elutes in two peaks and the majority of the peroxidase elutes in the same volume as the second phosphorus peak. Very little protein appears to be associated with the first phosphorus peak since the protein : phospholipid ratio in fraction 7 is 107 μg peroxidase per μmol phospholipid, whereas the ratio in fraction 11, the major fraction of the second phosphorus peak, is 1679 μg peroxidase per μmol phospholipid. The major result of peroxidase association with phosphatidylserine small unilamellar vesicles, whether by nonspecific or covalent binding, appears to be the formation of a protein-lipid complex of lower molecular weight than the small unilamellar vesicles which the lipid initially formed. We have not investigated whether these particles enclose an internal aqueous space, but it seems likely that they do not.

Binding to phosphatidylcholine/stearylamine solvent evaporation vesicles. We investigated the binding of peroxidase to solvent evaporation vesicles, since they are a more useful vesicle for encapsulation and morphological studies than

TABLE II

THE BINDING OF PEROXIDASE TO 10:3 PHOSPHATIDYLCHOLINE/STEARYLAMINE SOLVENT EVAPORATION VESICLES

6 μmol lipid were reacted with 10 mg enzyme. The vesicles were separated from non-bound enzyme by sedimentation at 400 000 × *g* for 1 h (four successive washes).

Experiment	Protein:lipid ratio (μg protein/μmol phospholipid)
Control	3.8
Sample	65 ± 2 *

* Mean of three experiments ± S.D.

small unilamellar vesicles. Phosphatidylcholine/stearylamine solvent evaporation vesicles were used for this work, since we were unable to prepare solvent evaporation vesicles from phosphatidylcholine/phosphatidylethanolamine mixtures. Table II shows the amount of activated or activated and reduced peroxidase which will bind to 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles under coupling conditions. Binding is only appreciable when covalent attachment can occur, and the amount which binds per μmol phospholipid is approx. 25% of the amount which will bind to small unilamellar vesicles under similar conditions. Table III shows the result of coupling peroxidase to solvent evaporation vesicles, which contain entrapped [^{3}H]inulin. Comparison of the inulin : phosphorus ratio in the coated and uncoated vesicles reveals that 71% of the entrapped inulin is retained within the vesicles during the attachment of peroxidase to the liposome membrane.

Location of the peroxidase. In all experiments with small unilamellar vesicles and solvent evaporation vesicles where vesicle-associated enzyme activity has been measured, the peroxidase has not appeared to be sequestered within the liposomes since the addition of Triton X-100 to disrupt the vesicles does not cause any increase in the observed level of activity. This is illustrated in the first part of Table IV and suggests that the enzyme is exclusively located on the external surface of the vesicles. The results in the second part of Table IV show that the liposome membrane still presents a barrier to the free passage of perox-

TABLE III

THE EFFECT OF PEROXIDASE COUPLING TO 10:3 PHOSPHATIDYLCHOLINE/STEARYLAMINE SOLVENT EVAPORATION VESICLES ON ENTRAPPED [^{3}H]INULIN

[^{3}H]Inulin was entrapped in reverse phase solvent evaporation vesicles (REVs) and the vesicles were separated from non-entrapped [^{3}H]inulin by four successive sedimentations. 6 μmol lipid were then coupled to 10 mg peroxidase and further purified by centrifugation.

Sample	[^{3}H]Inulin:phosphorus ratio (cpm/μmol)	% retention
Uncoated REVs	59 870	100
Coated REVs	43 099	70

TABLE IV

THE LATENCY OF PEROXIDASE ASSOCIATED WITH 10:3 PHOSPHATIDYLCHOLINE/STEARYLAMINE SOLVENT EVAPORATION VESICLES

Protein:phospholipid ratios were determined from the nitrogen and phosphorus content.

Location of peroxidase	Protein:lipid ratio (μg protein/μmol phospholipid)	Enzyme activity:phosphorus ratio (units/μmol)		% latency
		Triton X-100 present	Triton X-100 absent	
Covalently attached	67	11.21	11.27	0
Entrapped	13.84	8.27	2.43	71
Entrapped and covalently attached	55.24	6.83	3.65	47

idase substrates when coated with peroxidase. In this experiment peroxidase was entrapped in 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles to which peroxidase was then attached. These vesicles should have peroxidase both entrapped within them and attached to their external surface. The uncoated vesicles (enzyme inside only) exhibit 70% enzyme latency, which suggests that some passage of the substrate occurs across the positively charged membrane. As expected, the coated vesicles exhibit reduced but detectable latency, which shows that some peroxidase is still entrapped and the coated vesicle membrane presents a partial barrier to the passage of the peroxidase substrate. This further confirms that peroxidase attached to vesicles that are normally impermeable to peroxidase is located exclusively on the external surface of the vesicles.

Activity of peroxidase. Table V shows the specific activity of peroxidase in two experiments after the various stages of activation and coupling. A 60% loss of activity during coupling has most frequently been observed in these experiments. Loss of activity appears to be associated with fluorodinitrobenzene

TABLE V

THE EFFECT OF VARIOUS TREATMENTS ON THE SPECIFIC ACTIVITY OF PEROXIDASE

Samples were subjected to the treatment shown and all treatments shown for the samples above them in the table. n.d. = not done, n.m. = activity not measured.

Treatment	Expt. I		Expt. 2	
	Specific acitivity (units/mg)	% of original	Specific activity (units/mg)	% of original
None	647	100	861	100
Fluorodinitrobenzene	436 **	67	n.m. *	—
HIO_4 + $(CH_3OH)_2$	425	66	719	84
Coupled				
liposome bound	n.d.	—	377	44
liposome non-bound	279	43	394	46

* Fluorodinitrobenzene blocking performed at pH 8.1.

** Fluorodinitrobenzene blocking performed at pH 9.5.

TABLE VI

THE LOSS OF ACTIVITY OF PEROXIDASE MIXED WITH PEROXIDIZED VESICLES

5 ml of peroxidase in succinate buffer pH 6.0 were mixed with 1 ml of solution indicated below. The vesicles used were a 6 μmol/ml suspension of 1:2 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles.

Peroxidase mixed with	% of loss activity after 140 h
Buffer	16
Peroxidized vesicles	87
Peroxidized and reduced vesicles	0

treatment, with prolonged incubation at pH 9.5, and with $NaBH_4$ treatment. This finding is contrary to the observations of Nakane and Kawaoi [4] but has consistently been observed in this study. No preferential loss of activity of liposome-associated peroxidase occurs under oxygen-free conditions. However, a very rapid loss of peroxidase activity can occur if peroxidase-coated 1 : 2 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles are allowed to stand under air at room temperature. The rate of loss is variable but can amount to a total inactivation of the enzyme in 20 h. Table VI illustrates that the likely cause of the inactivation is peroxidation of the phospholipid fatty acyl chains. In this experiment samples of peroxidase were mixed with buffer, or well-peroxidized phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles or well-peroxidized vesicles which had been reduced with $NaBH_4$. The only sample in which appreciable reduction of activity is observed is that treated with peroxidized liposomes. The inactivation is slower than that observed when the peroxidase is attached to the lipid membrane, presumably because the enzyme is in less intimate contact with the peroxidized phospholipid. This inactivation of peroxidase appears to involve the degradation of the haem group since it is accompanied by a loss of the Soret absorption peak at 403 nm. The prime cause of the inactivation appears to be peroxidized phosphatidylethanolamine since it has not been observed with peroxidase attached to phosphatidylcholine/stearylamine vesicles.

Morphology. Fig. 5 shows the appearance of negatively stained peroxidase-coated phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles. Their appearance is much the same as uncoated vesicles since the peroxidase molecule is not large enough to be visible by negative staining. In some of our early experiments lower protein : lipid ratios were used for coupling peroxidase to liposomes, and polymerization of vesicles, shown in Fig. 6, was observed. Such polymerization was eliminated by increasing the protein : lipid ratio and increasing the reaction volume. Fig. 7 shows the appearance of positively stained and sectioned peroxidase-coated phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles that have been stained with diaminobenzidine and H_2O_2 for peroxidase activity. The diameter of these small unilamellar vesicles is about one quarter the thickness of the section and they consequently appear as small black patches of diaminobenzidine stain with no discernible membrane, which makes it impossible to define

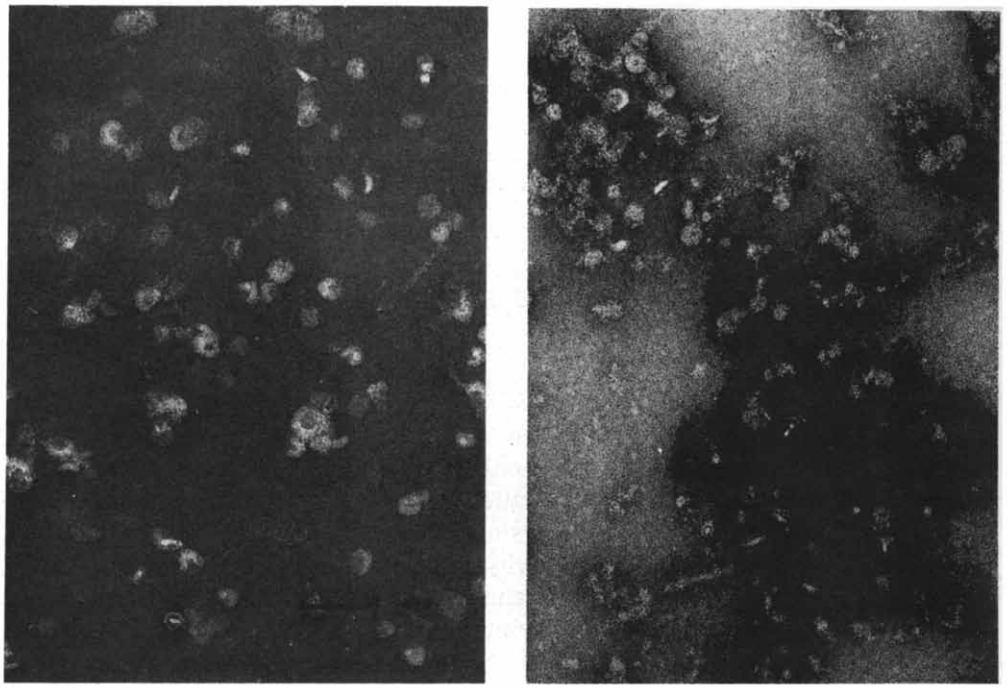
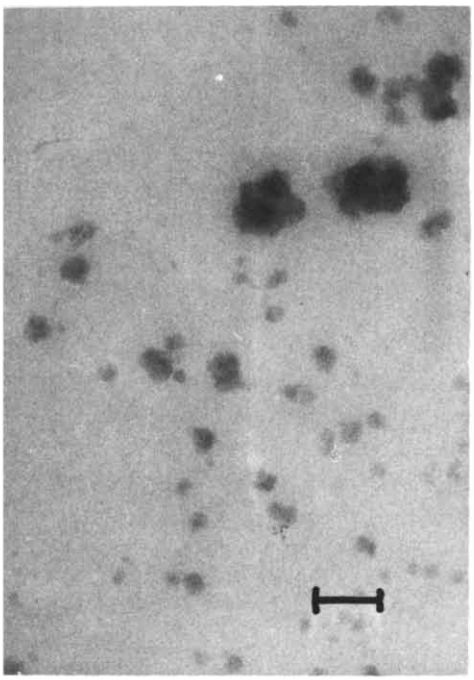


Fig. 5. Peroxidase-coated 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles (bar = 0.1 μm).

Fig. 6. Peroxidase-coated 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles negatively stained. In this preparation vesicles have become visibly aggregated (bar = 0.1 μm).

morphologically the location of the small unilamellar vesicle-associated peroxidase. Such a limitation does not apply to solvent evaporation vesicles and Fig. 8 shows the appearance of a negatively stained preparation of such vesicles which appear as 'floppy' uni- or oligo-lamellar structures. The appearance of negatively stained solvent evaporation vesicles correlates well with that seen in positively stained section of the same preparation (Fig. 9), which show a very similar size distribution and clear 'tramline' images of the liposome membranes. Peroxidase-coated solvent evaporation vesicles are shown in Fig. 10 and the liposome membrane with an external coat of diaminobenzidine stain can clearly be seen. Peroxidase is apparent neither on the internal surface of the membrane nor entrapped within the vesicles. Fig. 11 shows the appearance of phosphatidylcholine/stearylamine solvent evaporation vesicles that have peroxidase entrapped within them, and demonstrates the accessibility of the interior of the vesicles to diaminobenzidine stain and the complete absence of membrane staining when the enzyme is not specifically bound to the membrane. Vesicles that have peroxidase entrapped within them and bound to the outer surface of their membrane stain completely black and show no definable structure (not illustrated). If coated and uncoated solvent evaporation vesicles are mixed, two distinct populations of vesicle are visible in section (Fig. 12) which emphasizes



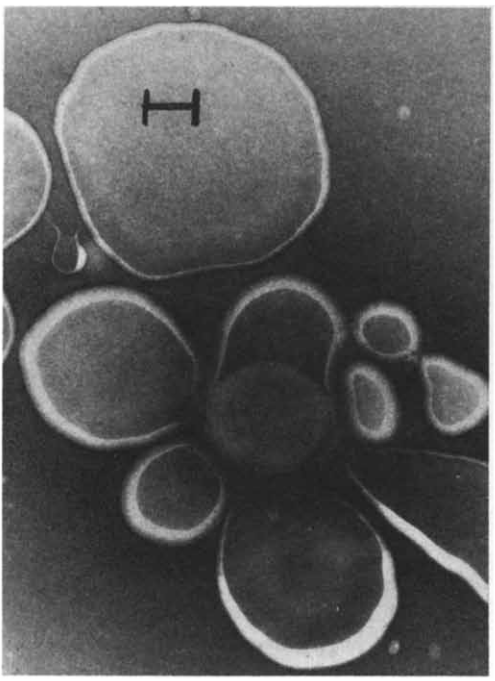


Fig. 7. Peroxidase-coated 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles stained for peroxidase activity. The vesicle structure cannot be seen, and they appear as dark patches (bar = 0.1 μm).

Fig. 8. 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles negatively stained (bar = 0.1 μm).

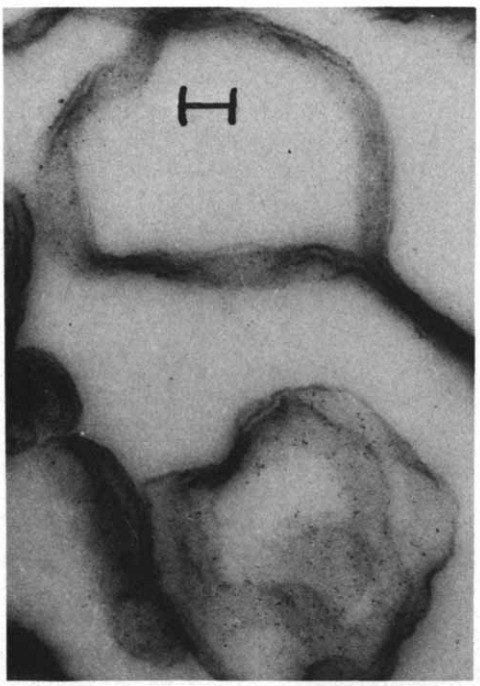


Fig. 9. 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles positively stained and sectioned (bar = 0.1 μm).

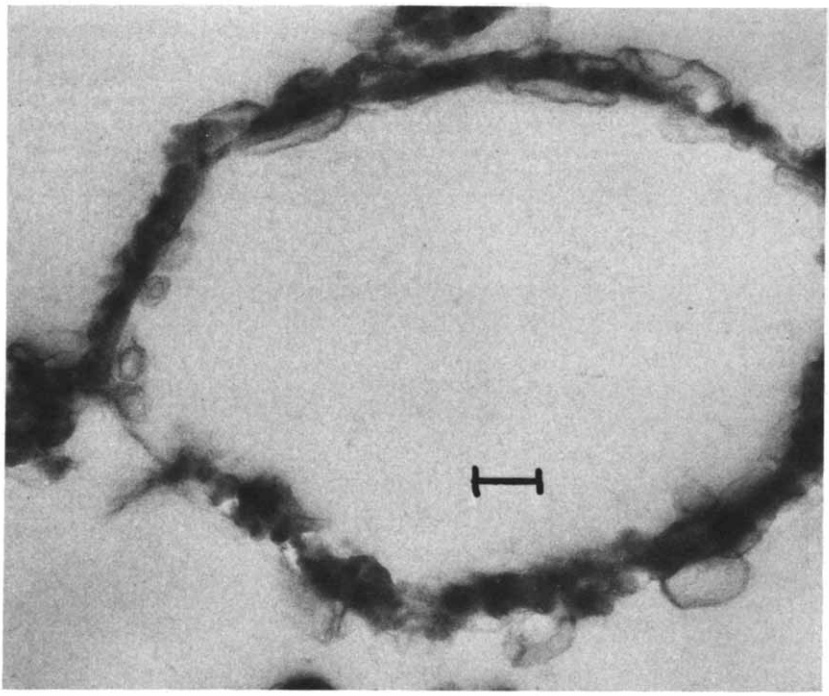


Fig. 10. Peroxidase-coated 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles. Stained for peroxidase, positively stained and sectioned (bar = 0.1 μm).

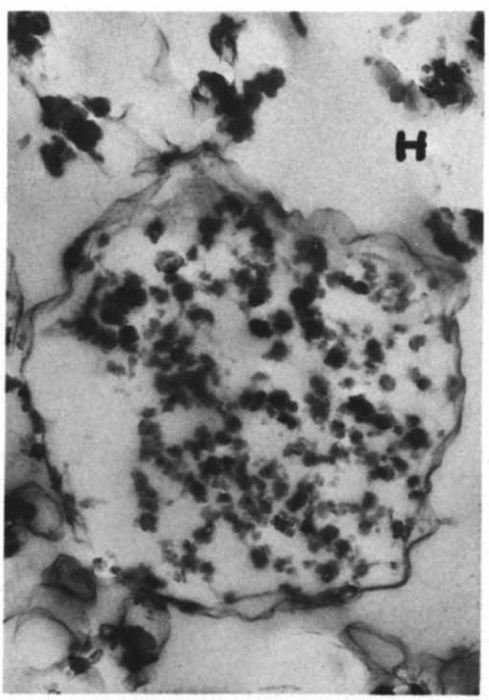


Fig. 11. Peroxidase encapsulated within 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles. Treated as for Fig. 10 (bar = 0.1 μm).

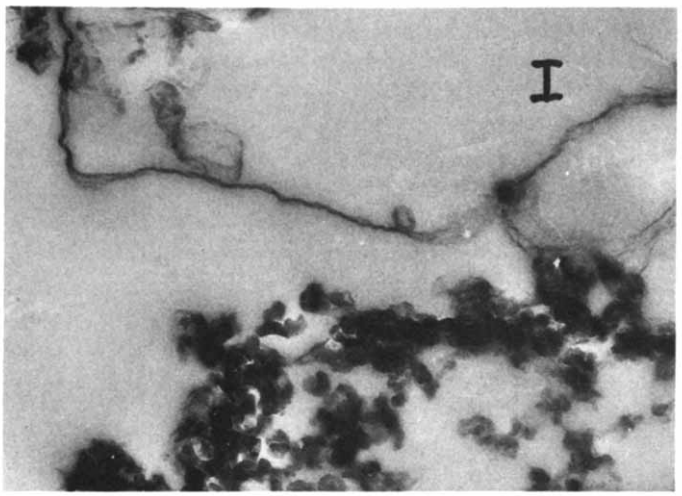


Fig. 12. 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles uncoated and coated vesicles mixed. Treated as for Fig. 10 (bar = 0.1 μm).

that the peroxidase is firmly bound and not free to exchange from one vesicle to another.

Interactions of vesicles with mouse peritoneal cells. In order to demonstrate the usefulness of peroxidase-coated vesicles for studying vesicle-cell interactions, we have examined using electron microscopy their interaction with mouse peritoneal cells in the mouse peritoneum. Peroxidase-coated vesicles appear to interact mainly with macrophages. There is little evidence of peroxidase staining of lymphocytes, erythrocytes or mast cells. Coated phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles rapidly associate with the plasma membrane of macrophages (Fig. 13) indicating either surface adsorption of the vesicles or a fusion of the vesicles with the cell membranes. The size of these vesicles does not permit distinction between the two processes on morphological criteria. At 1 h after injection, the peroxidase has become internalized within phagocytic vesicles, and by 6—24 h (Fig. 14) it is visible within larger phagocytic vesicles, presumably secondary lysosomes. Although peroxidase is known to be phagocytosed by cells, we have not observed a comparable reaction in similar experiments when a 100-fold greater concentration of free horseradish peroxidase was used.

Peroxidase attached to phosphatidylcholine/stearylamine solvent evaporation vesicles also rapidly associates with the macrophage membrane (Fig. 15) which must be due either to a fusion of the vesicles with the plasma membrane or to the transfer of the peroxidase molecules from the liposomes to the cell surface. Solvent evaporation vesicles are sufficiently large to observe surface adsorption of vesicles, and this does not appear to occur. On some cells, depressions in the membrane can be seen (Fig. 16) in which large amounts of peroxidase appear to be accumulating as if prior to shedding or endocytosis. At 1 h after injection no peroxidase can be found associated with any of the cells in the peritoneal wash which may indicate either that the macrophages have shed

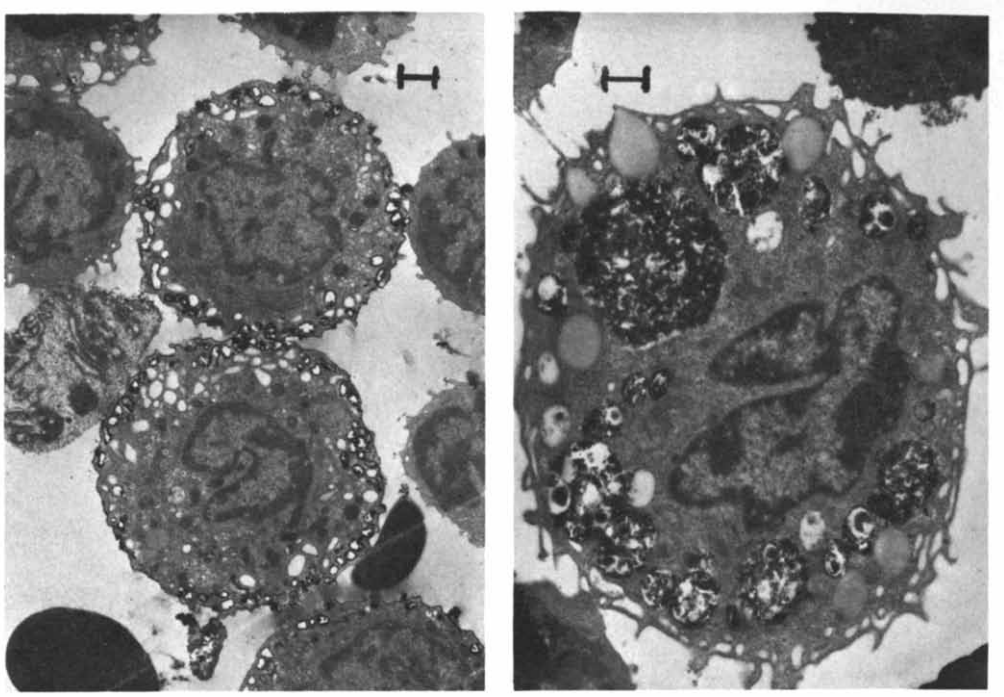


Fig. 13. Mouse peritoneal cells, washed from peritoneum 1 min after injection of peroxidase-coated 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles (bar = 1 μm).

Fig. 14. Peritoneal cells 24 h after injection of peroxidase-coated 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles (bar = 1 μm).

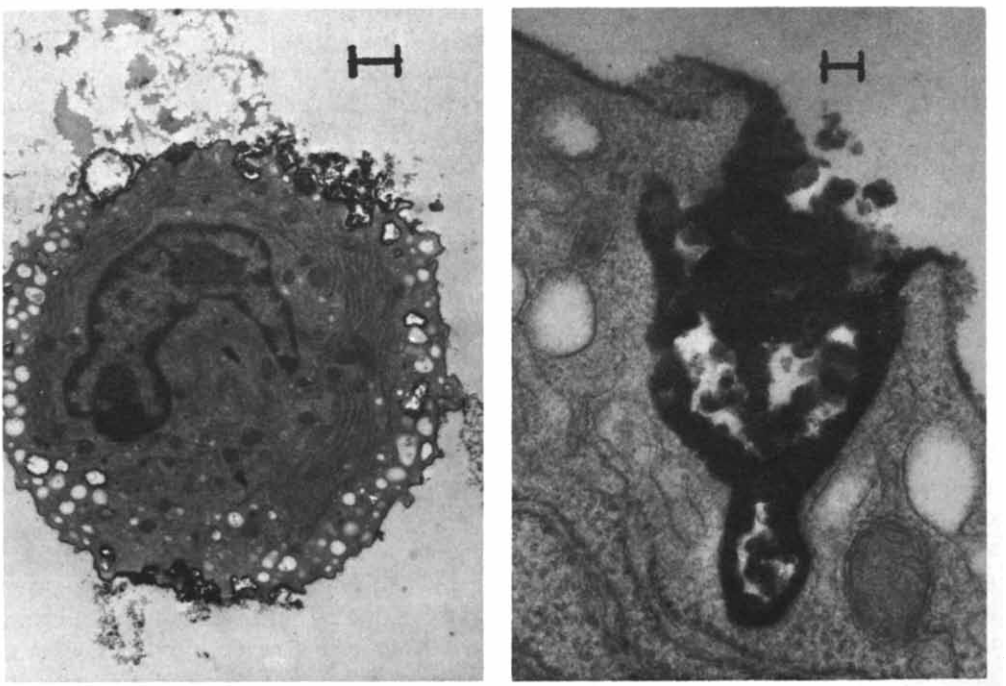


Fig. 15. Mouse peritoneal cell 1 min after injection of peroxidase-coated 10 : 3 phosphatidylcholine/stearylamine solvent evaporation vesicles (bar = 1 μm).

Fig. 16. As in Fig. 15 at higher magnification (bar = 0.1 μm).

the material or that they have lysed. It is unlikely that the cells would have degraded the enzyme in this short time, since in experiments with coated phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles, peroxidase-labelled cells were observed up to 24 h after injection of the material.

Discussion

The coupling of peroxidase to liposomes using the method of Nakane and Kawaoi occurs by the formation of a Schiff base from the primary amino group of phosphatidylethanolamine, stearylamine or phosphatidylserine and the aldehyde groups created on the oligosaccharides of peroxidase by periodate oxidation. The unstable Schiff base is converted to a more stable secondary amine by $NaBH_4$ which also reduces residual aldehydes to alcohols. It is impossible to predict the efficiency with which this reaction will couple peroxidase to vesicles, since this depends upon many factors including the concentration of reactants, the number of reactive groups available, and the efficiency of the Schiff base formation. However, on the basis of simple mass-action considerations, one may qualitatively predict that increasing the initial peroxidase : vesicle ratio should increase the amount of binding to the vesicles unless saturation of the vesicle surface has been achieved. Saturation of 2 : 1 phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles appears to occur when 250 μg peroxidase/μmol phospholipid are bound to vesicles. Moreover, doubling the phosphatidylethanolamine content of phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles, which, in spite of the asymmetric distribution of phosphatidylethanolamine in the small unilamellar vesicles [13], would double the outer surface phosphatidylethanolamine content of the vesicles, has no significant effect upon the amount of peroxidase bound per μmol lipid when 10 mg peroxidase interact with 4 μmol lipid. We therefore conclude that phosphatidylcholine/phosphatidylethanolamine vesicles are maximally coated with peroxidase when 250 μg peroxidase/μmol phospholipid are bound to them, even though unreacted phosphatidylethanolamine molecules may be present in the outer vesicle surface.

Under such conditions, peroxidase molecules may be closely packed on the vesicle surface thereby preventing the interaction of other peroxidase molecules with unreacted phosphatidylethanolamine molecules. We have calculated a theoretical value of 1167 μg/mol for maximal coating of small unilamellar vesicles from the available physical data (see Appendix). One possible reason for the difference between the theoretical and experimental values is that peroxidase may not pack on the liposome surface as a perfect sphere, when linked to the lipid by multiple bonding with its carbohydrate residues.

The amount of peroxidase bound per μmol lipid to phosphatidylcholine/stearylamine solvent evaporation vesicles is about one quarter of the amount bound to phosphatidylcholine/phosphatidylethanolamine small unilamellar vesicles under similar conditions. Such solvent evaporation vesicles might couple less peroxidase if the primary amino group of stearylamine is less reactive than that of phosphatidylethanolamine when incorporated into a phosphatidylcholine bilayer. The amount bound may also be reduced if the solvent

evaporation vesicles are not completely unilamellar; electron microscopic examination of the solvent evaporation vesicles does in fact show some oligolamellar structures.

The binding of peroxidase to phosphatidylserine vesicles has striking consequences which require further investigation. The transformation of small unilamellar vesicles into particles of much smaller size, possibly micellar in form, may reflect the inability of the liganded phosphatidylserine to form bilayer vesicles. The coupling of peroxidase to phosphatidylserine should not alter the net charge of the phospholipid head groups, since the primary amino group of the phosphatidylserine molecule is converted ultimately to a secondary aliphatic amine which should exhibit a pK_a at least as high as that of the original primary amino group. However, the initial interaction of peroxidase with phosphatidylserine will produce an imine which is weakly basic and will leave the phosphatidylserine molecule with two negatively charged groups, which may cause disruption of the vesicle structure. Whilst further investigation is required to elucidate this phenomenon, phosphatidylserine would appear an unsuitable molecule to use in coupling peroxidase to vesicles.

The preparation of peroxidase used for this study is reported to contain two basic isoenzymes which is confirmed by the isoelectric focusing pattern for the unmodified enzyme. The reduction of the isoelectric points of the isoenzymes by reaction with fluorodinitrobenzene is to be expected, since fluorodinitrobenzene will abolish the basicity of lysine residues by converting them to secondary aromatic amines. Fluorodinitrobenzene reaction at pH 8.1 is known to result in an incomplete substitution of dinitrophenyl groups on the peroxidase-lysine residues [4] and gives a very heterogenous product. Fluorodinitrobenzene reaction at pH 9.5 appears to give five well defined products, as judged by their isoelectric points. One might expect exhaustive fluorodinitrobenzene substitution to give only two bands since the original preparation contains two isoenzymes. The appearance of five may indicate some heterogeneity of the major isoenzyme which does not focus to a sharp band even at low loading. The reduction of peroxidase activity by fluorodinitrobenzene blocking and borohydride reduction has consistently occurred in our experiments. Nakane and Kawaoi have observed only a reversible inhibition of activity by fluorodinitrobenzene due to F^- and no permanent inactivation with 30 times the concentration of $NaBH_4$ which we have used. This discrepancy may arise from differences in experimental technique, though this seems unlikely since peroxidase is extremely resistant to inactivation. A more interesting possibility is that peroxidase may have been inactivated for the substrate with which we have measured its activity (2,2′-azino-di(3-ethylbenzthiazolinesulphonate) [6], but not for the substrate which Nakane and Kawaoi used (*m*-cresol). The inactivation of peroxidase by peroxidized liposomes is not unexpected since DNA is known to be similarly inactivated [14] and peroxidized fatty acids have been shown to inactivate proteins [15]. The inactivation of proteins involves the oxidation of thiols, though it is clear that haem groups are readily oxidized by peroxidized linoleic or arachidonic acid [16]. Only polyunsaturated fatty acid peroxides appear able to oxidize proteins in this way, which may explain why of the lipids we have used only egg phosphatidylethanolamine appears to inactivate peroxidase. Egg phosphatidylethanolamine

generally contains appreciable quantities of linoleic acid whereas egg phosphatidylcholine contains very little. The inactivation of peroxidase by peroxidized lipid emphasizes the need for rigorously maintaining anaerobic conditions when entrapping proteins in liposomes containing unsaturated phospholipids.

Electron microscopic observation of peroxidase-coated vesicles provides confirmation that the vesicles are still intact and clearly demonstrates for solvent evaporation vesicles that the peroxidase is located on the external surface of the intact vesicle membrane. The interaction of these vesicles with macrophages demonstrates that such an approach may provide much information on the interaction of vesicles with cells in vitro and in vivo. The use of peroxidase-coated vesicles with ferritin entrapped within them may permit the detailed morphological study of the fusion of vesicles with cells. Though coated vesicles do not appear to interact with cells in the same way as uncoated vesicles, it may still be possible to use peroxidase as a membrane marker for vesicles without modifying their surface properties by attaching it to the inner surface of the vesicles, which could be achieved by entrapping the peroxidase prior to initiation of coupling. The marked affinity of coated vesicles for macrophages is interesting, but requires biochemical characterization to establish the extent of macrophage uptake and the effect of metabolic inhibitors on the process.

Though antibody molecules are known to bind nonspecifically to liposomes [17], and can be sonicated into the liposomes membrane [18], covalent attachment should prove a superior method for antibody association with vesicles since the process should be more controllable and the product should prove more stable in the presence of serum proteins which could displace nonspecifically adsorbed immunoglobulins. This study has considerable implications for the possible antibody targeting of vesicles since it is the first demonstration that large amounts of protein may be covalently linked to the external surface of liposomes without causing their total disruption. The covalent attachment of antibodies [19,20] and pepsin [21] to liposomes has been previously described, but the amount of protein coupled was very small and not appreciably more than the nonspecific binding. Two approaches which may prove more generally applicable for the coupling of proteins to vesicles are the activated-thiol method [22] and the use of periodate-oxidized lipids. The activated-thiol method has been used to couple proteins under mild conditions and would require the synthesis of a modified lipid, possibly a phosphatidylethanolamine. Several lipids including phosphatidylglycerol, phosphatidylinositol or cerebrosides could be oxidized with periodate to generate aldehydes on their polar head groups. The coupling procedure would be identical to that used for peroxidase and would avoid the need to block primary amino groups on the protein. We are currently investigating the use of these methods.

Appendix

The Stokes radius (a) of peroxidase may be calculated from its diffusion coefficient (D) by the equation:

$$a = kT/D6\pi\eta$$

where k = Boltzman constant ($1.381 \cdot 10^{-16}$ erg/°C), T = absolute temperature and η = viscosity of the solvent ($1 \cdot 10^{-2}$ P for pure water at 20°C). The diffusion coefficient for horseradish peroxidase at 20°C in pure water is $7.05 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ [23] which gives a Stokes radius of 3.044 nm. If we assume that peroxidase molecules may be attached to the liposome surface with no intervening spaces, then the radius of packing will be equal to the sum of the radius of the liposome and the protein (15.544 nm). The area of packing will be 3036 nm^2, and the cross-sectional area of the peroxidase molecule is 29.1 nm^2. Hence, 104 molecules of peroxidase may be packed around a 25 nm small unilamellar vesicle. Assuming $1.7 \cdot 10^4$ small unilamellar vesicles/μmol lipid and a molecular weight of 39 780 for peroxidase, this corresponds to 1167 μg peroxidase per μmol phospholipid.

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