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of Doctor of Philosophy, with a major in Biochemistry.

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USE OF LIPID VESICLES TO
TRANSFER BIOMOLECULES INTO CELLS

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ABSTRACT

This research project involves the use of lipid vesicles as carriers to deliver bioactive molecules either into the cells or onto the cell membrane.

Large unilamellar vesicles (LUV), made of egg yolk lecithin (EYL), were demonstrated to be endocytosable by Chinese hamster V79 cells and therefore could be used as lysosomotropic agents for drug or enzyme delivery. Two cytochemical techniques were developed to provide evidence to support endocytosis of these vesicles.

When V79 cells were allowed to incubate with LUV containing ^{125}I -radiolabeled lysozyme, the lipid uptake was twice as fast as the vesicle-content uptake. Among the trapped-content uptake, 22.3% were inhibited by cytochalasin B or a combination of NaN_3 and deoxyglucose. Chloroquine did not inhibit the uptake. These results suggested that about 22.3% of the trapped-content uptake might be due to endocytosis.

When cells were treated with LUV containing horse-radish peroxidase (HRP), cytochemical studies revealed that peroxidase-positive particles were enclosed in vacuoles inside the cells. The morphology of the deposited reaction product was distinctly different from that resulted from pinocytosis of free HRP. These observations indicated that lipid vesicles were endocytosed by the cells.

When cells were treated with LUV containing 5-Br,

4-Cl, 3-indolyl phosphate (BCIP), a substrate for lysosomal acid phosphatase, electron microscopy revealed precipitated granules inside the cells. This precipitate formation was inhibited when chloroquine or NaN_3 together with deoxyglucose were included in the incubation medium. The formation of the electron-dense reaction product located in the lysosomes would specifically indicate the breakdown of BCIP after the vesicles had been endocytosed and delivered to the lysosomes.

In summary, endocytosis took place when Chinese hamster V79 cells were incubated with LUV. HRP and BCIP are suitable entrapped markers for the endocytotic activity. LUV that have high trapping capacity can be potentially used as carriers to deliver bioactive molecules into cells by an endocytotic mechanism.

Acetylcholine receptor (AChR) from Torpedo californica was attempted to be transferred to L cell membrane by lipid vesicles. Cholate solubilized AChR were reconstituted with or without the Sendai virus envelope proteins into azolectin lipid vesicles by Sephadex G50 column chromatography. When the suspended L929 cells were incubated with vesicles containing only AChR (AChR vesicles), about 2.8×10^4 AChR molecules/cell became associated with the cells. A two fold increase was observed when cells were treated with vesicles containing AChR and Sendai virus envelope proteins (AChR-VEP vesicles). The turnover of the AChR on the cell surface were rapid in both cases.

Indirect immunofluorescent data showed distinct difference in the distribution of AchR on the cell surfaces. Every cell that was treated with AchR-VEP vesicles could still be labeled after 40 hr of culture, while most AchR vesicle-treated cells could not. Immunoferritin EM studies suggested that AchR-VEP vesicles fused with cells resulting in the insertion of AchR into the cell membrane, while AchR vesicles only adsorbed on the cell surface. Complement-mediated cytotoxicity data also supported these results. AchR-VEP vesicle treatment resulted in 12% of the cell lysed after antibody-complement fixation by anti-Sendai antibody, which was comparable to those treated with native Sendai virus. When anti-AchR antibody was used for the assay, the cytotoxicity for AchR-VEP vesicle-treated cells was 2.87 fold higher than those treated with AchR vesicles. Since the viability of the cells will only be affected by specific antibody binding to antigens in the membrane, the cytotoxicity data could be interpreted as that AchR molecules were introduced into cell membrane more efficiently by AchR-VEP vesicles than by AchR vesicles.

In summary, lipid vesicles containing Sendai envelope proteins can be used as carriers to introduce AchR into the plasma membrane of L cells. Possibilities of further studies of the functional properties of AchR in L cells were discussed.

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LIST OF ABBREVIATIONS

LUV	large unilamellar vesicles
SUV	small unilamellar vesicles
MLV	multilamellar vesicles
EYL	egg yolk lecithin
HRP	horseradish peroxidase
BCIP	5-Br, 4-Cl, 3-indolyl phosphate
AchR	acetylcholine receptor
Ach	acetylcholine
^{125}I - α BGT	α -bungarotoxin radiolabeled with ^{125}I
HN	HN glycoprotein of Sendai virus
F	F or fusion protein of Sendai virus
HAU	hemagglutination unit
PMSF	phenylmethyl sulfonayl fluoride
VEP	viral envelope proteins

PART A

NOVEL MARKERS FOR ENDOCYTOSIS

OF LIPID VESICLES

BY CULTURED CHINESE HAMSTER V79 CELLS

CHAPTER I

INTRODUCTION

Interaction of lipid vesicles with cells have been studied by many investigators (see reviews 1, 17 and 18). Demonstrated mechanisms of interaction include fusion, endocytosis, adsorption and lipid exchange (17). In particular, endocytosis of lipid vesicles offers the opportunity of using lipid vesicles as lysosomotropic carriers for enzymes and drugs. Various means to control and even to enhance the endocytosis of lipid vesicles by cells have been attempted.

The present research project involves the use of lipid vesicles to deliver bioactive molecules inside the cells. Large unilamellar vesicles (LUV) were chosen to be the carriers because of their high trapping efficiency and, therefore, they were expected to be more efficient for the delivery.

Preliminary work has been done to elucidate the mechanisms of vesicle-cell interactions of these LUV with V79 cells (1). These results suggested that, when cells were treated with vesicle preparation in medium without divalent cations, Ca^{2+} and Mg^{2+} , most of the vesicles were not taken up as intact structures. The present report is to demonstrate the lysosomotropic property of LUV in the presence of Ca^{2+} and Mg^{2+} ions and the usefulness of this vesicle preparation

as potential carriers for lysosomal delivery.

Two histochemical techniques were developed to provide strong evidence to support the endocytosis of these lipid vesicles.

Lipid Vesicles and Their Medical Applications

Structurally, there are three principal types of lipid vesicles. Multilamellar (MLV), small unilamellar (SUV) and large unilamellar (LUV) vesicles. Multilamellar vesicles are most commonly prepared by hydrating and swelling phospholipids in aqueous solution. These vesicles are composed of a large number of closed concentric phospholipid bilayers. They are of different size up to 10 μm in diameter (79). Sonication of MLV results in the formation of SUV. The size of these SUV range from 200 to 500 $\text{\AA}$. and they consist of a single lipid bilayer. SUV can also be prepared by injecting an ethanolic solution of phospholipid through a small needle into an aqueous solution (80). Several techniques have been developed for the preparation of large unilamellar vesicles (LUV). Papahadjoulos et al. have described a reverse phase evaporation method for preparing LUV, which involves the fusion of phosphatidylserine SUV by Ca^{2+} and subsequent removal of Ca^{2+} by EDTA (81). Deamer and Bangham (19) developed an ether-injection method for LUV formation. They are unilamellar and are about 0.2 μm in diameter. Such LUV can trap approximately ten times more material than SUV.

Lipid vesicles have been developed over the past ten years both as model systems for the study of cell membranes and as possible vehicles for directing drugs to specific targets in the body. The physical properties of lipid vesicles which has been studied for a long time are now proving essential to the study of their interaction with cell membranes. The molecular organisation, lipid mobility and other properties are extremely relevant to their therapeutic applications. Several of the physical properties of lipid vesicles can be varied at will: Size can be adjusted from 200Å for SUV up to several microns for MLV. Negative or positive charge can be imposed by the incorporation of charged phospholipids. Control of permeability to the entrapped substances and the stability is also feasible by the addition of cholesterol or other sterols into the vesicle membrane. Finally, manipulation of the surface characteristics of lipid vesicles will enable them to recognise specific cells.

Lipid vesicle membrane encloses an aqueous internal space. By the use of simple techniques, it can accommodate a wide variety of water soluble substances. Alternatively, lipid soluble materials, including the hydrophobic macromolecules such as membrane proteins, can be inserted into the lipid bilayers.

The interaction of phospholipid vesicles with cells has been studied extensively. In vitro study showed different modes of vesicle-cell interaction, such as fusion, adsorption and endocytosis. Uptake experiments of SUV, made of egg

yolk lecithin by Chinese hamster lung fibroblast, showed that at 37°C, vesicle-cell fusion was the predominant mechanism (2, 3 and 82). The study using 3T3 mouse fibroblasts showed vesicle uptakes both by vesicle-cell fusion and by endocytosis (83). In the fluid state, negatively charged lipid vesicles favor uptake by vesicle-cell fusion, while neutral fluid vesicles and negatively charged solid vesicles favor uptake by endocytosis. Magee and coworkers (84) observed that cationic lipid vesicles adsorbed and endocytosed by cultured HeLa cells. The authors suggested that positively charged vesicles initially interact with cells via adsorption and subsequently internalized by endocytosis. These studies indicate that the modes of vesicle-cell interaction depend entirely upon the physical states (such as the charge and fluidity) of the vesicle membrane.

Lipid vesicles were first considered as possible therapeutic carriers by Gregoriadis and Ryman (85) in the treatment of storage diseases. Amyloglucosidase containing MLV were intravenously administered to rats. A large proportion of these lipid vesicles was taken up by the liver and spleen and were localized predominantly in the lysosomes. When a patient with Type II glycogen storage disease was treated with these vesicles, a decrease in size of the liver was observed. L-asparaginase containing vesicles injected intraperitoneally has been used successfully to suppress an implanted L-asparagine-dependent lymphosarcoma in C3H mice

(86). In vitro studies showed different mechanisms for vesicle-cell interaction. Such mechanisms, however, may be less relevant for the in vivo system. For instance, fusion has been seen in the absence of serum. Components such as α_2 -macroglobulin might adhere to the lipid vesicles and promote phagocytosis. Nevertheless, the possibility that mechanisms could be selectively induced to enhance the access of vesicle-entrapped drugs to a particular target organelle was proposed (88). The exciting prospects of medical applications ranged from the treatment of tumors, enzyme and hormone deficiencies, metal storage diseases, arthritis to immunopotentialiation and interferon induction (89).

The reason why lipid vesicles were proposed as suitable drug carriers is based on the following observations. Lipid vesicles made of phospholipid are nontoxic, biodegradable and immunologically inert. They can protect the encapsulated drugs from immunological reactions. Other beneficial effects from these vesicles as carriers include reduced dosages, delayed drug elimination and increased cellular permeability either by endocytosis or vesicle-cell fusion.

The use of lipid vesicles as carriers has provided a suitable means to reduce drug losses and undesirable side effects. The shortcoming of this method is that distribution of these vesicles is not selective. They are transported mostly to the liver and spleen. A method for targeting

lipid vesicles would vastly expand their potential. Attempts have been made to improve the target selectivity. A promising approach is to incorporate immunoglobulins raised against given cells into the lipid vesicles. The immunoglobulins direct the vesicles to the desired cell, where the drug expresses its effects. Such a delivery system has been successfully tested in vitro. Significantly, immunoglobulin coated lipid vesicles have delivered entrapped hexosaminidase A into Tay-Sachs leukocytes more effectively than the uncoated vesicles. Conversely, the free enzyme was not taken up by the cells. Some degree of selectivity has also been achieved using lipid vesicles associated with desialylated fetuin for in vivo study (90). Desialylated fetuin, which exhibits affinity for hepatic parenchymal cells, incorporated onto the lipid vesicle surface was found to mediate the uptake of the vesicle associated bleomycin by the appropriate cell type.

From the survey, it is clear that the use of lipid vesicles as drug carriers is well established. This is an exciting and highly significant area of research. The studies described above may be summarized as follows: (1) Lipid vesicles may encapsulate drugs or macromolecules, such as enzymes; (2) In vitro and in vivo administration of enzymes by means of lipid vesicles increases the cellular uptake relative to the uptake of free enzyme; (3) The mechanisms of vesicle uptake depends upon the properties of the vesicles, such as charge, size and fluidity; (4) The association of vesicles to cells may be enhanced by coating the vesicles

with ligands recognizable by surface receptors of the cells;
(5) Targeting of lipid vesicles to specific tissues is,
at the present time, only partially successful.

CHAPTER II

MATERIALS AND METHODS

Preparation of Large Unilammellar Vesicles

The ether-evaporation method of Deamer and Bangham (19) was used with modification to prepare large unilammellar vesicles (LUV). Twenty mg of egg yolk lecithin (EYL) was dissolved in diethyl ether at a concentration of 1.6mg/ml. The phospholipid/ether solution was injected at a rate of 0.2ml/min into 2-4ml of Gey's solution without Ca^{2+} and Mg^{2+} but containing an aqueous marker. The aqueous solution was kept at 48°C by a water jacket and was gently stirred with a magnetic stirrer. After injection, the liposome suspension was filtered through a Millipore filter (pore size 1.2um) to remove any large or clumped vesicles. The filtrate was chromatographed on a Sepharose 2B column (1 x 30cm) to remove residual ether and untrapped marker. The vesicles eluted at the void volume fractions were diluted and used for uptake experiments.

Vesicle Uptake

Chinese hamster V79 cells were grown as monolayer culture on Falcon plates (35mm) in Lact medium containing 10% fetal calf serum as described previously (3). Cultures of near confluency were washed three times with Gey's balanced salt solution (3) and vesicle suspension (1mg lipid

per ml) was added. Incubation at 37°C was carried out for various periods of time. After incubation, the cells were washed three times with Gey's solution and detached from plates by incubating in Puck's EDTA solution for 10 min at 37°C. The suspended cells were washed two more times with Gey's solution by centrifugation. The final cell pellets were solubilized in 1% Triton X-100 and the total cell-associated radioactivity was determined by liquid scintillation counting.

Electron Microscopy-Cytochemistry

Horseradish peroxidase was entrapped in the vesicle internal space at 20mg/ml. Free, untrapped enzyme was removed by gel filtration on Sepharose 2B. After vesicle-cell incubation, the cells were washed three times with ice-cold Gey's solution and then fixed with 2.5% glutaraldehyde in 0.2M Na phosphate buffer, pH7.2 for 10 min at 4°C. The cells were rinsed three times with 0.1M Tris buffer, pH7.2 and stained with the diaminobenzidine reagent of Graham and Karnovsky (20) which contained 0.3mg/ml 3,3'-diaminobenzidine tetrahydrochloride in 0.1M Tris buffer, pH7.2 and 0.01% H₂O₂. The reaction was allowed to proceed for 30 min at room temperature. Excess reagent was removed by three washes with Tris buffer. The cells were scrapped off the plate by a rubber policeman and pelleted in a microfuge tube. Cells were postfixed with 2% OsO₄ in 0.2M Na phosphate, pH7.2 for 2 hr at 4°C. They were then dehydrated through

a graded series of aqueous acetone and finally embedded in Epon. Ultrathin sections without further staining were viewed by using a Zeiss or JEM6C electron microscope. In controls, cells were treated with free horseradish peroxidase (20mg/ml) and processed for EM cytochemistry identically as described above.

In another series of experiment, 5-Br, 4-Cl, 3-indolyl phosphate (BCIP) was used as a vesicle-entrapped marker at a concentration of 2mg/ml. Free, untrapped BCIP was removed by gel filtration. After incubation for 1 hr at 37°C with vesicles containing BCIP, cells were washed and processed for transmission EM as described above. Ultrathin sections were viewed without further staining. Three types of control were done: (1) cells were incubated with free BCIP (2mg/ml) in Gey's solution; (2) cells were fixed with 1% glutaraldehyde for 10 min at 4°C, washed, and then incubated with free BCIP (2mg/ml); and (3) cells were preincubated with a combination of NaN_3 (30mM) and deoxyglucose (50mM), or with chloroquine (100uM) in Gey's solution for 30 min at 37°C, and then incubated with BCIP containing vesicles for 1 hr at 37°C in the presence of the drug. In all cases, cells were washed after incubation and processed for EM identically as described above.

Other Methods

Lysozyme was radioiodinated as followed: To 1mg of lysozyme in 10ul of 0.5M Na phosphate buffer, pH7.2 was added 0.5mCi carrier free Na¹²⁵I. The reaction was started by adding 5ul chloramine-T (1mg/ml) and allowed to proceed for 60 min at room temperature. After incubation, 5ul sodium metabisulfate (1mg/ml) was added to stop the reaction. The mixture was loaded on a Biogel P-4 column (1 x 20cm) and eluted with divalent cation free Gey's solution. The void volume fractions containing labeled lysozyme were pooled and diluted for vesicle trapping. The specific radioactivity of ¹²⁵I-lysozyme was 1×10^7 cpm/mg protein. Protein determination was done by the Lowry method (21).

CHAPTER III

RESULTS

Sensitivities of Lipid and Trapped-content Uptakes to Inhibitors

In order to follow the uptake of the lipid and the trapped content of the lipid vesicles by cells, LUV were doubly labeled with ^{125}I -lysozyme as trapped-content marker and ^3H -EYL for the lipid. Typical uptake curves of these two components by Chinese hamster V79 cells at 37°C for different periods of time are shown in Figure 1.

In the presence of divalent cations, Ca^{2+} and Mg^{2+} , the kinetic and amount of lipid uptake appear to be similar to the lipid uptake when the cells were incubated with lipid vesicles in the absence of these divalent cations as previously reported (1), but the amount of trapped content uptake was increased by 1.95 fold. In this case, the data showed that the lipid uptake was twice as fast as that for the trapped-content uptake. After 1 hr of incubation, the amount of trapped-marker uptake only represented 45.5% of the theoretical amount if 100% represents the amount of trapped-content uptake at a rate equals to that of the lipid uptake.

In the case in which divalent cations were excluded from the incubation medium, only 13% of the theoretical value was observed. This discrepancy has been addressed

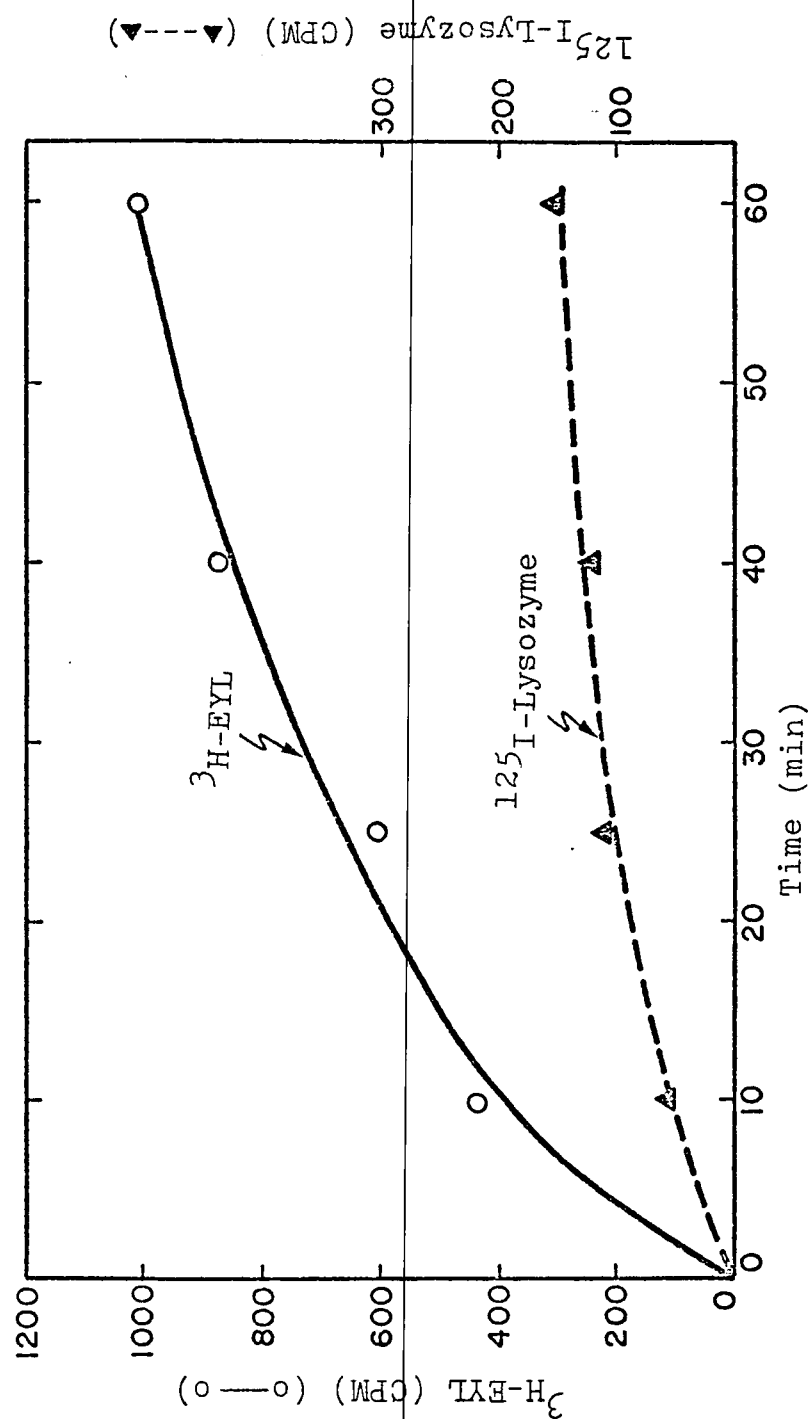


Figure 1. Uptake of ^3H -EYL and ^{125}I -lysozyme by V79 Cells treated with ^{125}I -lysozyme-entrapped LUV at 37°C . o—o, ^3H -EYL; ▲---▲, ^{125}I -lysozyme.

previously as that the bulk of the lipid was taken up by lipid transfer or leaky fusion. This study showed that the trapped-content uptake was increased by 1.95 fold with the inclusion of divalent cations. This increase of the trapped-content uptake can be the result of: (1) stabilization of lipid vesicles adsorbed onto the cell surface, (2) induction of endocytosis in the presence of Ca^{2+} and Mg^{2+} , or (3) the enhancement of the fusion process by these divalent cations in such a way that the trapped content released into the cells was increased.

Parameters such as size, charge or fluidity of lipid vesicles have been shown to determine, to some extent, the mode of interaction between the lipid vesicles and the cells (8-13). Small unilamellar vesicles, made of EYL, have been shown to interact with V79 cells by the fusion process (3). In non-leaky fusion, vesicles fuse with the plasma membrane of the cell with the release of all the trapped content into the cells. This mechanism demanded that the rates for the trapped-content and vesicle-lipid uptake be the same. The present study showed that more than half of the vesicle lipid was not taken together with the trapped content. This observation disproved vesicle-cell fusion to be the major mechanism for these vesicles to interact with cells. By comparing the two systems, the size of the lipid vesicles seems to be an important factor that governs the way these vesicles interact with the cells.

How was the rest of the trapped content taken up by

cells? Lipid vesicles can become cell associated by stable adsorption to the cell surface, by endocytosis or by fusion with the cell membrane.

Endocytosis has been demonstrated to be dependent on metabolic energy (14 and 15) or membrane movement (16). Inhibitors can be used to cut off the energy supply or membrane mobility and therefore block endocytosis. The effects of different inhibitors on the lipid and trapped-content uptakes were shown on Table 1.

When the cells were incubated with the lipid vesicles containing ^{125}I -lysozyme in the presence of 0.1mM cytochalasin B, 30.2% of the lipid uptake was inhibited and the trapped-content uptake was similarly inhibited by 22.3%.

Similar inhibition was observed when the cells were simultaneously treated with sodium azide and deoxyglucose. The lipid uptake were inhibited by 32.4% and the trapped-content uptake, by 22.3%. No inhibition of either the lipid or trapped content uptakes when 100uM chloroquine or excess amount of non-radioactive lysozyme was included in the incubation mixture. These data suggested that a substantial fraction of the lipid vesicle uptake was dependent on membrane mobility and metabolic energy supply. The sensitivities of the trapped-content uptake to these inhibitors further implied that endocytosis was likely to be one of the mechanisms for vesicle-cell interaction.

Chloroquine, a potent inhibitor for lysosomal enzyme, did not affect the uptakes of the vesicle components. This

TABLE 1. Effects of Inhibitors on the Uptakes of ^3H -EYL and ^{125}I -lysozyme by V79 Cells Treated with LUV*

	Lipid Uptake		^{125}I -lysozyme Uptake	
	cpm	% of control	cpm	% of control
Control	1549	100.0	274	100.0
Cytochalasin B (0.1mM)	1082	69.8	213	77.7
NaN_3 (30mM) + deoxyglucose (50mM)	1048	67.6	213	77.7
Chloroquine (100uM)	1666	113.1	298	108.7
Lysozyme (1 mg/ml)	1608	103.8	282	102.9

* Incubation was done at 37°C for 1 hr.

observation agrees with other study that chloroquine does not inhibit endocytotic activities. The inclusion of non-radioactive lysozyme in the medium showed no effect on the uptake of the vesicle components. This clearly indicated that free lysozyme in the medium could not compete with the vesicle-entrapped lysozyme uptake. Furthermore, the free lysozyme also could not inhibit the uptake of vesicle lipid or lipid vesicles as a whole.

Cytochemical Localization of Vesicle-entrapped HRP in Cells

The localization of the entrapped marker in cells can give a lot of information of how the vesicles interact with the cells. Horseradish peroxidase (HRP) was chosen for this study because a sensitive and reliable cytochemical assay is available for ultrastructural localization.

When cells were treated with HRP-containing vesicles for 1 hr, numerous cytoplasmic inclusions were observed which contain peroxidase-staining materials (Figure 2). Many of these cytoplasmic inclusions appeared to be membrane bound and were located in the internal space of the vacuoles. The morphology was quite different from that of the peroxidase-reaction product located at the pinosomes after the cells had taken up the free HRP by pinocytosis. In that case, the peroxidase-staining materials were associated with the membrane of the vacuoles, while the internal spaces were not stained (Figure 3). These morphological differences suggested that the lipid vesicles containing the HRP were

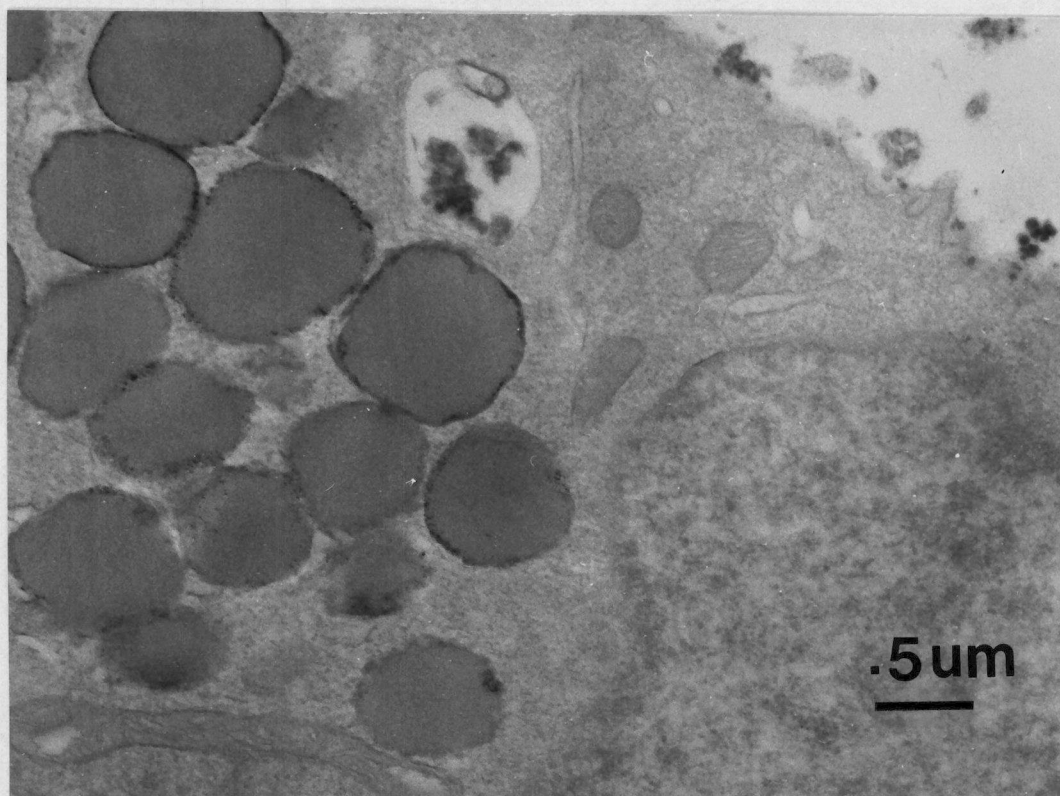


Figure 2. Transmission electron micrograph of V79 cells treated with LUV containing HRP for 1 hr at 37°C.

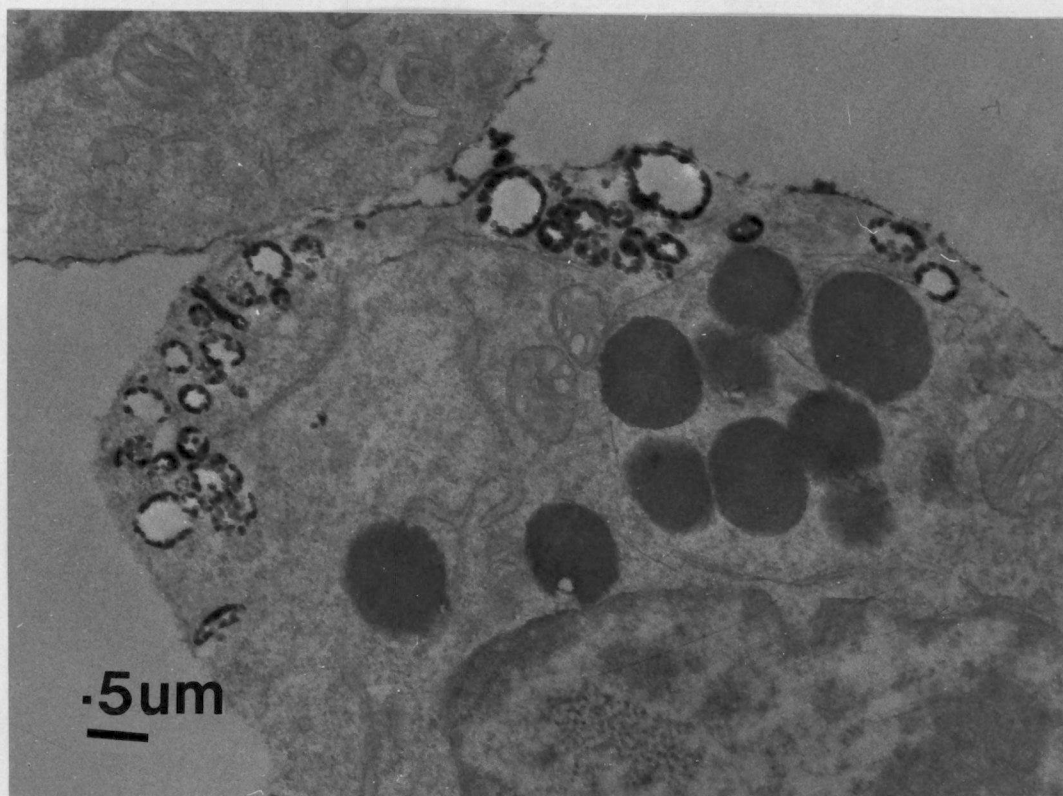
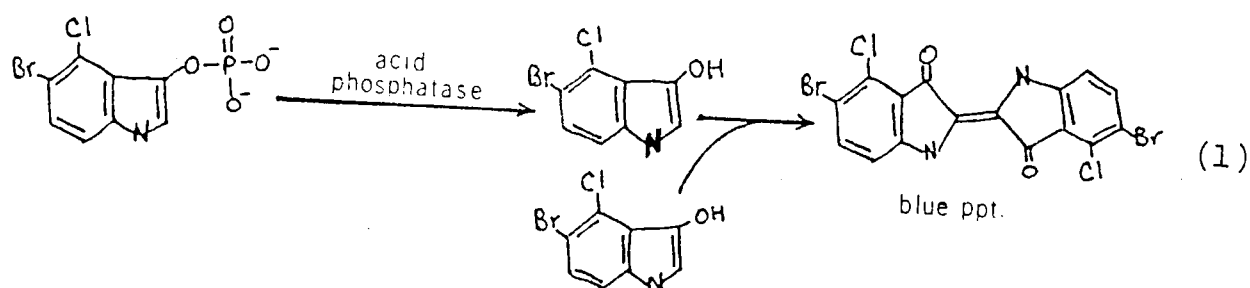


Figure 3. Transmission electron micrograph of V79 cells treated with free HRP for 1 hr at 37°C.

taken up by endocytosis. Some of the electron-dense particles remained at the cell surface which suggested vesicle adsorption. Electron-dense reaction product was also observed located at the periphery of the lipid droplets. This type of staining was not observed in cells treated with free HRP and therefore could not be derived from pinocytosis of free HRP or from endogenous peroxidase activity. Since the lipid droplets were derived from the lysosomes, therefore, the activity stain in these lipid droplets might imply the endocytosis of the lipid vesicles containing the HRP. Peroxidase-positive staining was not detected in control cells incubated in the complete diaminobenzidine reagent, indicating that endogenous cellular peroxidases did not contribute to the observed staining patterns.

Cytochemical Demonstration of Endocytosis by BCIP

After endocytosis of lipid vesicles, lysosomal enzymes would digest the vesicle membrane and release the trapped content in the lysosomes. If a compound that has been trapped in the lipid vesicles is a specific substrate for the lysosomal enzyme and forms reaction product that can be used for cytochemical assay after the reaction, then identification of this reaction product in the lysosomes can be used as direct evidence to demonstrate endocytotic activity of the lipid vesicles.

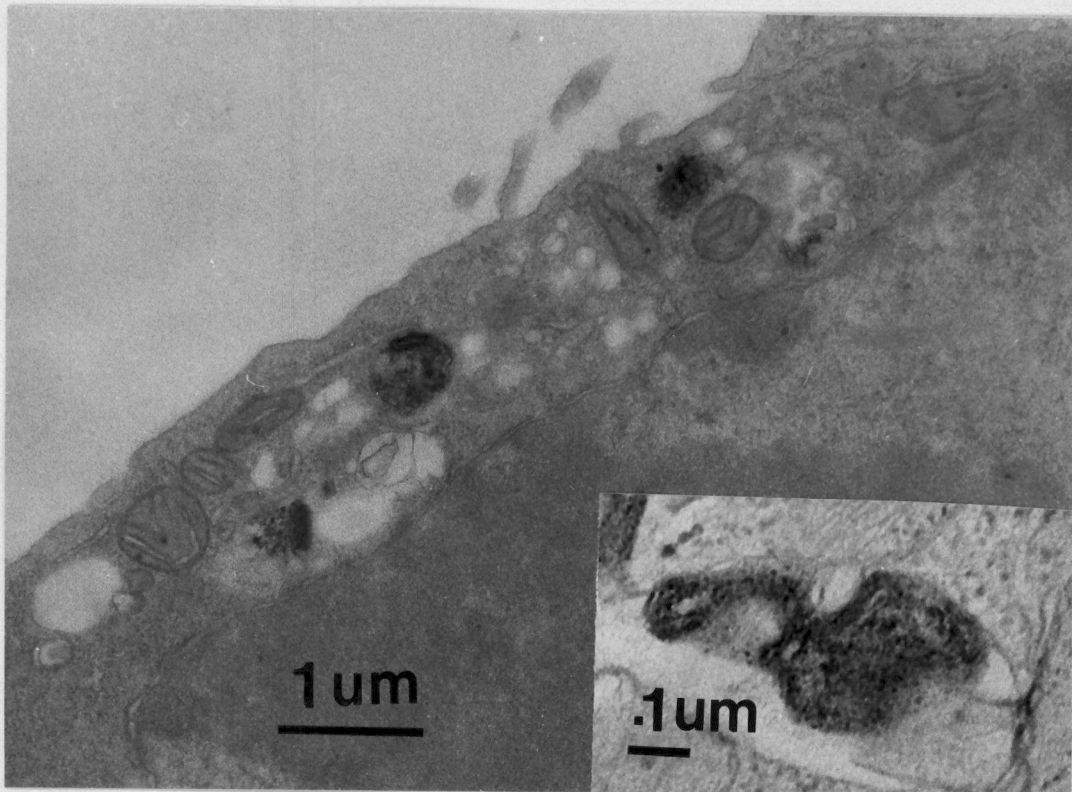


5-Br, 4-Cl, 3-indolyl phosphate (BCIP) is particularly suitable for this study. It can readily be hydrolyzed by acid phosphatase which is a specific lysosomal enzyme. The enzymatic reaction is shown above. After enzymatic hydrolysis, the nature of the final reaction product has very fine grains and is nondiffusible. It can also be observed by electron microscopy. Therefore, this substrate is particularly suitable for cytochemical study.

After the cells had been incubated with lipid vesicles containing BCIP for 1 hr at 37°C, they were washed and processed for electron microscopy. Electron-dense cytoplasmic inclusions were observed by transmission electron microscopy (Figure 4,a). These inclusions were membrane bound, indicating that the lipid vesicles were endocytosed and digested inside the cells, such that the entrapped marker was released and broken down in the secondary lysosomes, presumably by lysosomal acid phosphatase.

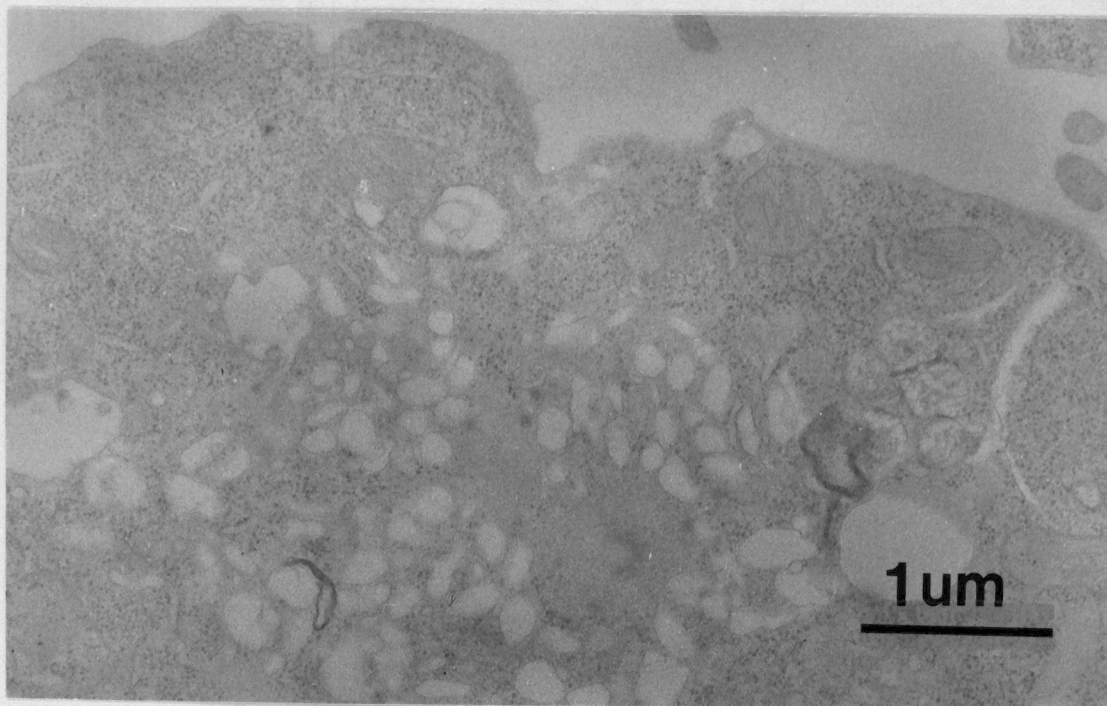
These reaction products were not detected when the cells were incubated with the BCIP-containing lipid vesicles in the presence of chloroquine or a combination of NaN_3 and deoxyglucose (Figure 4, b and c). Chloroquine is a potent

Figure 4. Transmission electron micrographs of V79 cells treated with LUV containing BCIP for 1 hr at 37°C. (a) without any inhibitors; (b) with 30mM NaN_3 and 50mM deoxyglucose; (c) with 100uM chloroquine. 3

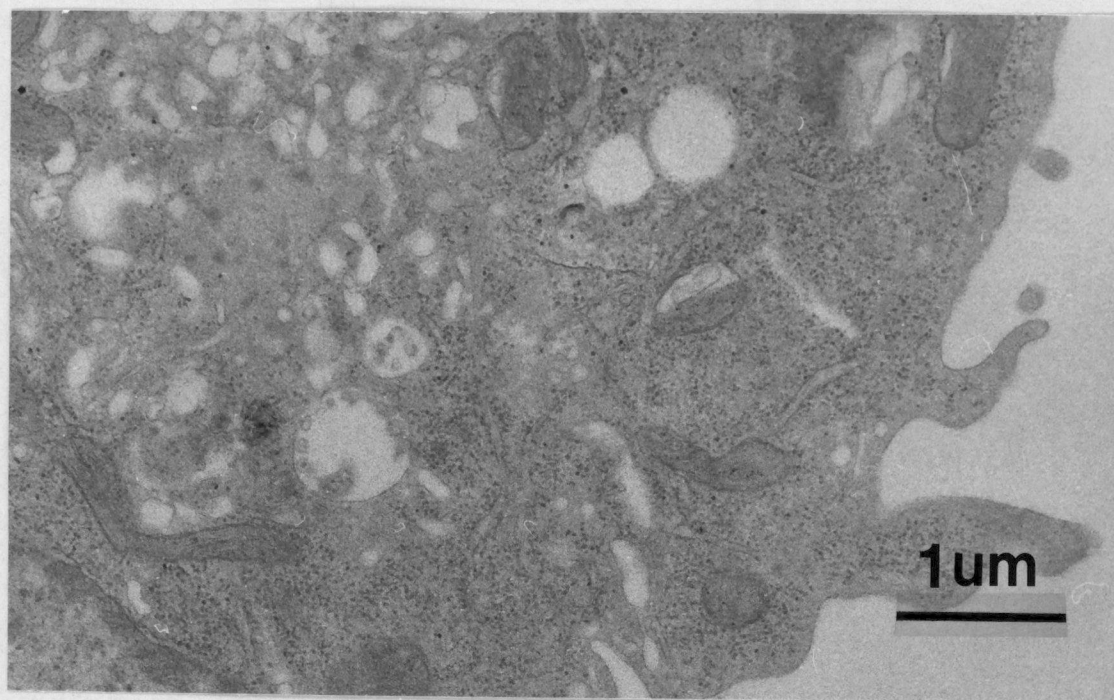


(a) without any inhibitors.

Figure 4 (continued)



(b) with 30mM NaN_3 and 50mM deoxyglucose.



(c) with 100uM chloroquine.

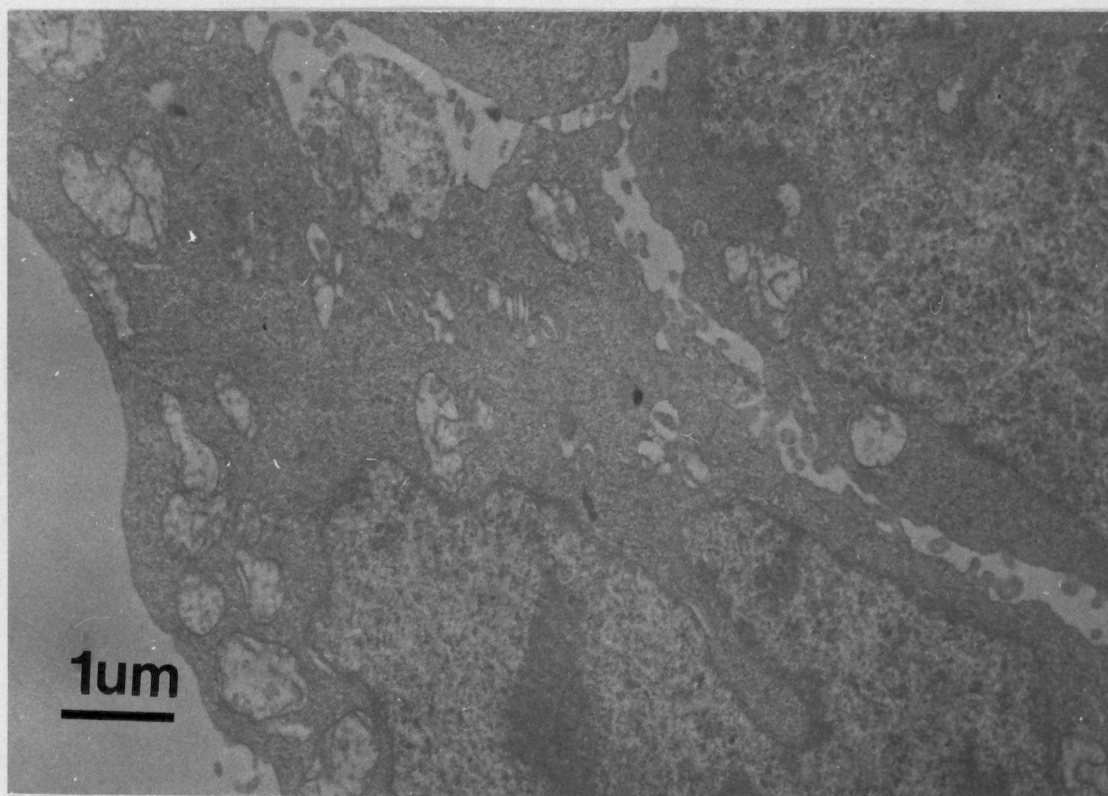
Figure 4 (continued)

inhibitor for the lysosomal enzymes, but it does not inhibit endocytosis. Previous study using ^{125}I -lysozyme as marker for the vesicle-trapped content indicated that chlorquine had little effect on the uptake of the trapped content (Table 1). This suggested that endocytotic activity was not inhibited. BCIP-reaction product was not detected in these cells further indicating the trapped content was not broken down, presumably due to the inhibition of the lysosomal enzyme by chloroquine.

Free BCIP at concentration of 2 mg/ml. induced cytotoxicity as indicated by the swelling of the mitochondria (Figure 5, a), which was not observed in control cells (Figure 5, b). No electron-dense reaction product was detected in these cells. This observation implied that even if BCIP leaked out from the lipid vesicles, it could not contribute to the staining pattern observed in those cells treated with BCIP-containing lipid vesicles.

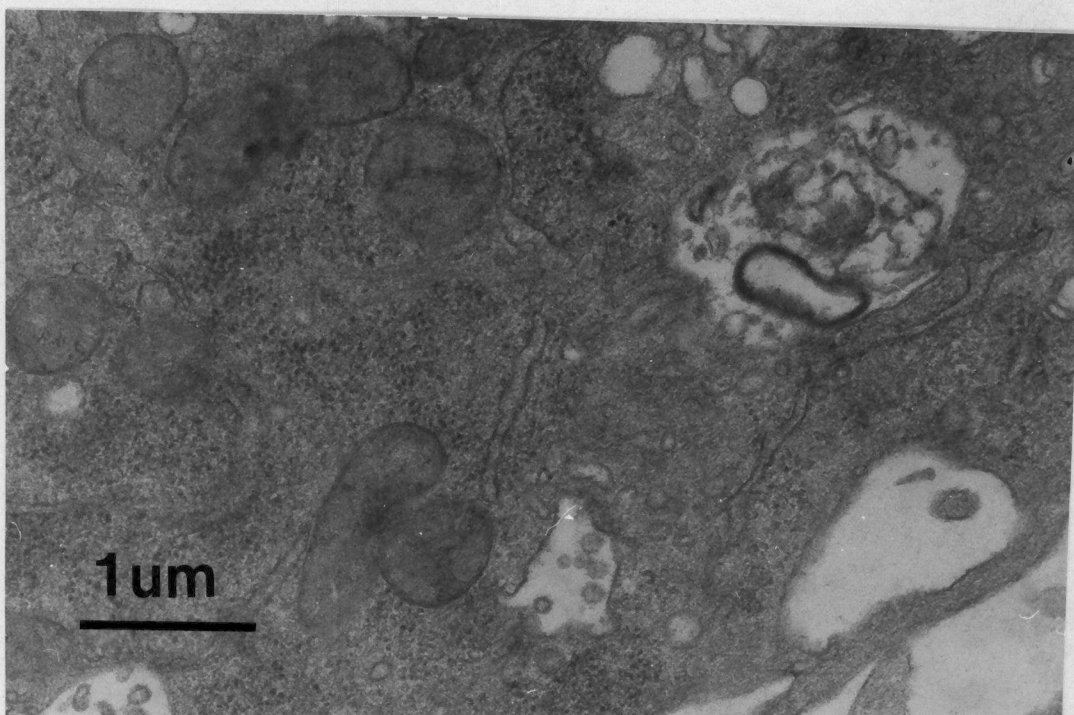
From these cytochemical studies, it has been clearly demonstrated that BCIP can be used as a novel cytochemical marker for endocytotic uptake of lipid vesicles. Since the reaction product would only be formed after the vesicles were endocytosed, quantitative measurement of endocytotic activity by BCIP product formation using spectroscopic technique may be valuable for further study.

Figure 5. Transmission electron micrographs of V79 Cells with or without BCIP treatment. (a) Cells were treated with free BCIP (2 mg/ml) for 1 hr at 37°C. (b) Cells were not treated with BCIP.



(a) Cells were treated with free BCIP (2 mg/ml) for 1 hr at 37°C.

Figure 5 (continued)



(b) Cells were not treated with BCIP.

Figure 5 (continued)

CHAPTER IV

DISCUSSION

Different approaches have been used to demonstrate endocytosis of LUV by Chinese hamster V79 cells. The results of these experiments with LUV made of egg yolk lecithin can be unambiguously interpreted as the demonstration of endocytosis. Three lines of evidence were presented as followed: (a) sensitivity of the uptake of vesicle-entrapped marker ^{125}I -lysozyme to metabolic energy inhibitors and cytochalasin B which are known to be potent inhibitors for endocytosis; (b) cytochemical localization of vesicle-entrapped HRP activity in vacuoles similar to secondary lysosomes; (c) ultrastructural demonstration of BCIP reaction product in cell vacuoles similar to lysosomes.

Two different cytochemical markers were used to locate the vesicle-trapped content inside the cells. HRP has a molecular weight of about 40,000. It can be trapped in the internal space of the vesicles without any difficulties. The enzyme also has high sensitivity and can be used for light and electron microscopy. It is an excellent marker to locate the trapped content associated with the cells. The mechanisms of vesicle-cell interaction has been identified using this entrapped marker was endocytosis and adsorption. Quantitative measurements to distinguish the two processes were, however, not possible.

The amount of entrapped content and lipid associated with the cells can be quantitatively measured by radioactivity. When lipid vesicles containing ^{125}I -lysozyme were incubated with cells, the lipid was taken up twice as much as the trapped content. Among the trapped-content uptake, 22.3% was sensitive to the metabolic inhibitors and cytochalasin B. Since these drugs inhibit endocytosis, this portion of the uptake can be interpreted as due to endocytotic process, which corresponds to 10.14% of the total vesicle uptake. This kind of interpretation suffers some drawbacks. Although NaN_3 and deoxyglucose inhibit ATP production by shutting off the respiratory and glycolytic pathways, they may have other cellular effects. The same is true for the interpretation of cytochalasin B sensitivity. These drugs may have other indirect effects on the amount of vesicle uptake.

In order to quantitate the amount of trapped content that was taken up by endocytosis, a way to measure the uptake of vesicle component that is unique to the pathway has to be obtained. BCIP is particularly suitable for meeting this aim. It is a slightly brownish, soluble compound and is readily hydrolyzed by acid phosphatase which is a specific lysosomal enzyme. After enzymatic hydrolysis, the reaction product is highly insoluble and nondiffusible (87) and can be used for cytochemical study. In the present study, BCIP was used as a vesicle-entrapped marker. It is a highly negatively charged compound and can be faithfully

trapped inside the vesicles. Leakage from the vesicles was nondetectable after incubation at 37°C for 2 hr. Another unique feature of BCIP is that it is an artificial substrate for acid phosphatase. No natural reaction product of similar nature could be found in the cells. Therefore, the demonstration of BCIP reaction product in the cells after treating with vesicles containing BCIP could be interpreted as the result of endocytosis of the vesicles. Furthermore, cells treated with free BCIP did not show any granules inside the cells, which also argued against the possibility that BCIP leaked out from the vesicles and was taken up by the cells.

The original design of this artificial substrate is for cytochemical study by light microscopy. This is because the reaction product has an intense blue color and can be readily observed by light microscopy. This reaction product is a derivative of indigo blue. It has a adsorption maximum at 627 nm when dissolved in dimethylsulfoxide and can be easily quantitated by spectroscopic means. Since the reaction product is unique for the endocytotic pathway, kinetics studies for the endocytosis of lipid vesicles by quantitative measurement of product formation is highly possible. However, V79 cells appear to have considerably low activity for endocytosis of EYL vesicles, therefore only qualitative results were reported. Other systems, such as macrophages, which have much higher ability to endocytose particulate materials, have to be considered to examine the kinetics of

endocytotic activity.

It is known that the sonicated unilamellar vesicles, made of EYL, interact with V79 cells mainly through fusion (3). In this study, LUV of the same lipid composition was shown to interact with the same cell type by other mechanisms. In spite of the large trapping capacity of LUV, the ability of trapped-content delivery of these vesicles seemed to be limited to the lysosomes. An ideal delivery system utilizing lipid vesicles as carriers might be visualized as one in which the vesicles could discharge their contents directly into the target compartment, such as the lysosomes or cytoplasm. Such a possibility seems quite feasible by the proper manipulation of the lipid composition. Several reports have shown that lipid vesicles may fuse with cell membrane and release the trapped content into the cytoplasm (2, 4 and 5). Gregoriadis and Buckland have shown that lipid vesicles containing invertase could alleviate a storage disease in tissue culture model (6). Other in vivo studies have shown that enzyme-containing lipid vesicles injected into rats were rapidly removed from the circulation mainly by the liver and to a lesser extent by the spleen and lung. The vesicle components were demonstrated to be mainly in the lysosomal fraction (7). These results suggested that the carrying of enzymes by lipid vesicles into lysosomes could be applicable to the treatment of storage diseases.

In many genetic disorders, particularly for different storage diseases, well documented enzyme defects are

expressed in lysosomes. It would seem of interest to attempt to replace the missing enzymes in such cells by relevant enzymes entrapped in lipid vesicles. Encouraging results from our experiments should provide stimulating interest for exploring the possibility of using lipid vesicles as carriers for therapeutic agents in the treatment of inherited enzyme deficiencies.

PART B

TRANSFER OF ACETYLCHOLINE RECEPTORS
TO L-CELL SURFACE MEMBRANES BY LIPID VESICLES
CONTAINING SENDAI VIRUS ENVELOPE PROTEINS

CHAPTER I

INTRODUCTION

Since a variety of hydrophobic membrane proteins and glycoproteins can be incorporated into vesicle membrane, fusion of vesicles with plasma membrane offers a potential method for introducing specific proteins into the plasma membrane of cultured cells. Sendai viral envelope is known to have high capacity to induce cell fusion. The fusion activity resides on the viral envelope proteins. To test the feasibility of this method, the use of Sendai envelope proteins to reconstitute into the lipid vesicles to serve as carriers to introduce acetylcholine receptor (AChR) into the plasma membrane of the L cells was examined. AChR is chosen for these studies since it can be reliably distinguished from other cell components and it has very specific function. The incorporation of this particular protein into the cell can presumably modify the cell membrane function.

The present report provides a useful methodology of the use of Sendai viral envelope proteins reconstituted into lipid vesicles as carriers to introduce hydrophobic membrane proteins into plasma membrane of cultured cells. The methods described here can also be used to obtain information on the functional properties of specific membrane proteins.

Acetylcholine Receptor

AchR is the most thoroughly studied neurotransmitter receptor. It is also the first neurotransmitter receptor to be isolated and purified to homogeneity. There is a large amount of research about the involvement of this receptor in synaptic interaction, the molecular events in receptor function as a transducer and the structural role of receptor molecule in synaptogenesis. The acetylcholine receptor plays a significant role in long-term nerve-muscle interactions. The presence of extrasynaptic receptors has been suggested to be a prerequisite to the formation of new synaptic connections between motor neurons and skeletal muscle fibers. The new technique developed using iontophoretic mapping with acetylcholine permits the rapid construction of detailed maps of acetylcholine sensitivity (42). It was shown that ingrowing axons induced new clusters to form the synapse. Synapses can form at the active growth cones within 3 hr of nerve-muscle contact. Furthermore, AchR is of special interest for it is an antigen in an autoimmune disease, myasthenia gravis.

AchR-enriched Membrane Fragments

The molecular nature of acetylcholine receptor has been well studied mainly using the electric organ of either electric ray or eel (23, 29 and 50). Recent progress in the purification of AchR-enriched membrane was achieved by subcellular fractionation of electric tissue. These

membrane fragments are highly enriched with AchR. About 40-50% of the total protein is AchR. These membrane fragments are separated from those rich in acetylcholineesterase or Na-K ATPase (23). When examined at the electron microscopic level, AchR-enriched membrane fragments are mostly in the form of vesicles, with densely packed cylindrical rosettes 60-80Å in diameter (22-24). These structures can be immunospecifically labeled by antibodies against AchR further supporting the idea that these rosette structures are the AchR molecules. X-ray diffraction studies of AchR-enriched membrane fragment showed that the protein present spanned the membrane, extending 55Å on one side of the bilayer and 15Å on the other (25-26). The transmembrane nature of the AchR is further shown by immunospecific labeling of the exposed cytoplasmic face of the right-side-out membrane vesicles by anti-AchR antibody (27).

The transmembrane nature of AchR can easily explain the function of these receptors as ion channels. Ion channels consist of two distinct functional components, a gating device which opens and closes the channel and a selectivity filter determining which ion may pass through. The post-synaptic ion channels at the neuromuscular junction are controlled by a neurotransmitter molecule released from the presynaptic membrane. This neurotransmitter, acetylcholine (Ach), specifically binds to the AchR located at the post-synaptic membrane. Binding of the Ach to these receptors

triggers the all-or-none opening of the channels through which Na^+ ions flow along their electrochemical gradient. The AchR contains two distinct sites, the Ach-binding site and the site for ion translocation.

Molecular Structure of AchR

The AchR has been extracted by detergents and purified from fish electric organ and vertebrate skeletal muscle in sizable amounts. It is an oligomer of 250,000-300,000 mol. wt. (28 and 29). SDS-polyacrylamide gel electrophoresis of AchR gives distinct bands of apparent mol. wt. as follows: 40,000, 50,000, 60,000 and 65,000 (28-31). The 40,000 component is the only one that can be affinity labeled by Ach analogue and therefore suggested to be the AchR protein carrying the Ach binding site (51 and 52). The role of other chains are unknown.

Multiple Affinity States of AchR for Ach

Equilibrium binding studies of AchR-enriched membrane with ^3H -Ach showed a striking heterogeneous population of AchR (54 and 55). The shape of the binding curve is often sigmoidal which indicates positive cooperativity of Ach binding (33). In the membrane at rest the AchR is present mainly at the low affinity state. Agonist binding causes an inter-conversion to the high affinity state. Dissolution of the membrane by sodium cholate modifies the shape of the Ach binding curve and the intrinsic dissociation constants vesicles (59 and 60).

Sendai Virus

Sendai virus has one of the best known activities for cell fusion. This virus is an envelope virus. The viral envelope enclosed the RNA genome, its associated nucleoprotein (NP) subunits and the polymerase (P). Several biological activities of this paramyxovirus are associated with the viral envelope. These include hemagglutinating, hemolytic and cell fusion activities. The viral envelope consists of a lipid bilayer. The external surface of the lipid bilayer is covered with spike-like projections which are made up of two glycoproteins, the HN and F proteins. The envelope glycoproteins of Sendai virus have now been successfully isolated by extraction with non-ionic detergents and individual glycoproteins separated by affinity chromatography. The larger of the two envelope glycoproteins, the HN protein, possesses both neuraminidase and agglutinating activities. The smaller envelope glycoprotein, the F or fusion protein, is involved in virus-induced cell fusion and hemolysis.

The ability of Sendai virus to induce cell fusion is associated with the F glycoprotein. The evidence comes from studies of the structural difference of this protein in virions grown in different host cell. Sendai virus grown in eggs (62), primary chick embryo fibroblast (63) and primary calf kidney cells (64) is able to induce cell fusion

of AchR without changing the total number of toxin binding sites. Three distinct populations of sites with K_d close to 30nM, 0.1uM and 1uM were obtained by Scatchard plot of the binding data. Triton X-100 stabilizes primarily the high affinity sites (56). Local anesthetics inhibit the pharmacological response to agonists in a noncompetitive manner. It is suggested that local anesthetics bind to a site distinct from the Ach-binding site (57). A preliminary estimate of one local anesthetic binding site for four Ach binding sites has been reported. Local anesthetics cause an increase of the affinity of the membrane-bound AchR for agonists. They cause a change of shape of the Ach binding curve from a sigmoid to an hyperbola. Conversely, agonists enhance the affinity of local anesthetics for local anesthetic binding sites. All these effects are lost upon solubilization of the membrane by detergents. The interaction between Ach binding site and local anesthetic site requires the integrity of the membrane structure. Affinity labeling by histrionicotoxin in detergent gives a protein band of apparent mol. wt. of 43,000. This protein has the local anesthetic binding property similar to the native membrane. It is suggested that this component carries a site for local anesthetics and is separated from the AchR. Further data showed that this protein can be extracted by alkaline treatment. The affinity column purified AchR, devoid of this 43,000 protein, showed agonist induced ion translocation when the AchR was reconstituted into lipid

of a wide variety of cell types and is also able to lyse erythrocytes. However, when the same strain of virus grows in established mammalian cell lines such as MDBK cells (65 and 66) or mouse L cells (67) the virions lack cell fusion and hemolytic activities and are also noninfectious. Comparison of the envelope glycoproteins in Sendai virions grown in embryonated eggs with those grown in MDBK or L cells has revealed that while the HN glycoprotein is similar, virions grown in MDBK and L cells lack the F protein found in egg-grown virions and instead contain large amounts of a higher molecular weight component, F_0 .

Recent studies by Scheid and Choppin (68) have shown that F glycoprotein is composed of two disulfide-linked polypeptides, F_1 and F_2 . Cleavage of F_0 results in the formation of two polypeptides that remain covalently linked to each other. In MDBK and L cells, proteolytic cleavage of F_0 to F does not occur and progeny virions are released from infected cells containing F_0 rather than F. Thus the presence of F_0 instead of F in these virions correlates with their inability to induce cell fusion. Scheid and Choppin proposed that the proteolytic cleavage results in a subtle conformational change in the F_0 precursor and that both F_1 and F_2 chains are necessary for the functional activity of the F protein. Cleavage of F_0 precursor to produce F can be accomplished by treatment of those virions containing F_0 precursor with 1-10 μ g/ml trypsin for 10 min at 37°C. The conversion of F_0 to F is accompanied by restoration of

infectivities, cell fusion and hemolytic activities (66 and 69).

The HN glycoprotein is mainly responsible for attachment of the virion to the cell surface. F glycoprotein spikes can be selectively digested from egg-grown Sendai virus by trypsin (20ug/ml for 1 hr at 35°C), leaving the HN spikes intact on the surface. These F-deficient particles exhibit normal hemagglutinating and neuraminidase activities, but their infectivity, cell fusion and hemolytic activities are markedly reduced. When the HN spikes are selectively removed by a fungal protease (70), the virions are devoid of hemagglutinating and neuraminidase activities and also lose their fusion activities. Temperature sensitive mutant isolated by Porter (71) has defect on HN synthesis at the nonpermissive temperature. Although the F glycoprotein is present, these virions are unable to induce cell fusion.

The attachment of Sendai virus to cell surface via HN envelope glycoprotein is an essential primary step in virus-induced cell fusion. The virus is believed to bind to neuraminidase-sensitive sialic acid residues on cell surface.

Mechanism of Virus-cell and Cell-cell Fusion

There is now a general consensus that cell fusion induced by paramyxoviruses and other lipid enveloped viruses require the fusion of virus envelope with the cellular plasma membrane. Differences in opinion remain, however,

about whether cell fusion occurs at the same time as fusion of virus particles with the plasma membrane or whether virus-cell fusion and cell-cell fusion occur as related, but separated processes.

Fusion between virus envelope and cellular plasma membrane is the normal mechanism by which Sendai virus infects cells. Virus attachment and subsequent steps of virus penetration and cell-cell fusion can be distinguished by different experiments. Apostolov et al. (72) and Knutton (73) have proposed that cell-cell fusion occurs when a virus particle in close association with the membranes of two different cells fuses simultaneously with the plasma membrane of both cells, permitting the virus to act as a connecting bridge between the two cells. Progressive enlargement of such bridge caused by cell swelling results in complete cell fusion.

Fusion of viral envelope with plasma membrane can be separated experimentally from the process of cell-cell fusion. Cytochalasin B (74) and tertiary amino local anesthetics (75) do not impair virus-cell fusion but both drugs effectively inhibit the subsequent step of cell-cell fusion. Similarly Maeda et al. (76) have found that 0.38M glucose or mannose or 0.25M sucrose inhibits cell-cell fusion but does not affect virus-cell fusion. These results indicate that virus-cell fusion and cell-cell fusion are separate events.

Reconstitution of Viral Envelope

Isolation of the membrane components and reconstitution of envelopes from them are useful methods for the investigation of the functions of the envelope proteins. Different non-ionic detergents have been used for solubilizing the viral envelope glycoproteins. These non-ionic detergents can selectively solubilize the two spike proteins without much contamination from the nucleocapsid and the matrix protein. The viral envelope can be reconstituted with either exogenous lipid mixture or viral lipid extract by dialysis. The reconstituted vesicles contain the HN and F proteins. Spikes were seen on both the inner and outer surfaces. In all cases, neuraminidase and hemagglutinating activities were associated in these reconstituted membrane particles, but in some cases cell-cell fusion could not be induced by the reconstituted membrane, but retain their hemolytic activity and ability to fuse to cells. HN and F proteins require lipid for their functions.

Sodium cholate had been used for the reconstitution of viral envelope (77). This method required the selective solubilization of HN and F glycoproteins in Triton X-100. The detergent is then exchanged with sodium cholate by sedimentation into a sucrose gradient containing 2% cholate. Reconstitution of lipid vesicles with HN and F glycoproteins as well as the phosphatidylcholine occurred upon removal of cholate by dialysis.

Membrane reconstituted from purified HN protein exhibited

hemagglutinating and neuraminidase activities, while reconstituted particles containing purified F protein exhibited hemolytic activities when wheat germ agglutinin was co-reconstituted with the F protein and lipid.

The fusion of reconstituted viral envelope with cells was supported by the cytotoxicity of cells after being treated with reconstituted viral envelopes containing fragment A of diphtheria toxin. Fragment A itself has no toxicity for living cells in culture medium. When the cells were treated with fragment A-containing lipid vesicles with spike proteins, most of the cells died upon further culture. It is suggested that fragment A was released into the cytoplasm as the result of vesicle-cell fusion, which caused cell death.

CHAPTER II

MATERIALS AND METHODS

Preparation of AchR-enriched Membrane

The method for purification of AchR-enriched membrane was modified from that of Reed et al. (23). The flow chart for purification is shown in Figure 6. About 100g of tissue from electric organs of *Torpedo californica* (Pacific Biomarine, California) was cut into small pieces and homogenized for 3 min at maximum speed with a Waring blender with 50ml of Buffer I (1M NaCl, 10mM NaP, 0.02% NaN₃ and 0.1mM PMSF at pH7.4) at 4°C under a nitrogen atmosphere. The membrane suspension was further homogenized with a Brinkman polytron at maximum speed for 1 min and was then passed through four layers of cheesecloth. Nuclei and cell debris were removed by low speed centrifugation at 5,000 rpm for 10 min with GSA rotor. The supernatant was centrifuged at 100,000 x g for 45 min. The pellet was resuspended and a trace amount of ¹²⁵I-αBGT was used to follow the AchR. The membrane was washed once with 50ml Buffer II (same as Buffer I except with 0.4M NaCl instead of 1M NaCl). The membrane was washed again with buffer without salt (Buffer III). The pellet was then resuspended in 20ml of Buffer III and then diluted 1:1 with 50% sucrose (v/v) in Buffer III. It was layered on six sucrose gradients (35-41% sucrose continuous gradient with 8ml of 41% sucrose as cushion). Centrifugation was performed at 25,000 rpm for 7 hr with SW27 rotor. The

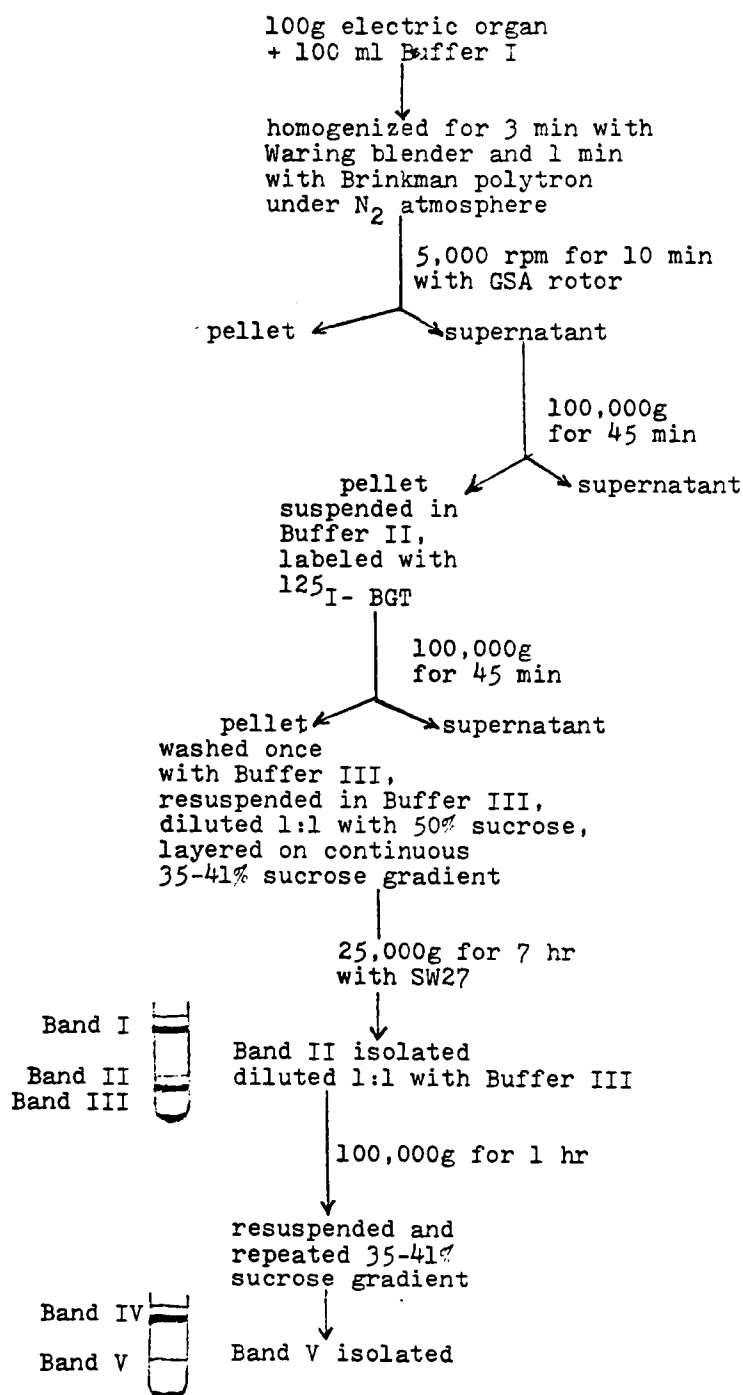


Figure 6. Purification scheme for AchR-enriched membrane from electric organs of Torpedo californica

middle membrane band (Band II), which had high toxin-binding activity, was pooled, diluted 1:1 with Buffer III and pelleted by centrifugation at 100,000 x g for 1 hr. These membrane fragments were suspended in 8ml of Buffer III and diluted with 50% sucrose. The sucrose gradient centrifugation of the nature were repeated as above. The middle band (Band V) was collected as the final AchR-enriched membrane fragments.

Sendai Virus

Sendai virus (Z strain) was grown in the allantoic sac of 10-day embryonic eggs inoculated with about 10^5 egg-infective doses. After 72 hr incubation at 37°C, the allantoic fluid was collected and centrifuged at 3,000 x g for 20 min. The virus in the supernatant was inactivated with a final concentration of 0.01% B-propiolactone overnight at 4°C and then at 37°C for 2 hr. The inactivated virus was purified by two cycles of differential centrifugation at 3,000 x g for 20 min and at 37,000 x g for 30 min. The purified virus was resuspended in BSS (140mM NaCl, 5.4mM KCl, 0.34mM Na₂HPO₄, 0.44mM KH₂PO₄, 1mM CaCl₂, 0.8mM MgCl₂, 10mM Tris-HCl and 1mg/ml glucose at pH7.4) at a concentration of about 3mg/ml.

Hemagglutination Titration

Hemagglutination test was carried out by Salk's pattern method. Five ul samples were assayed using microtiter plates with round bottom cups. Serial two-fold dilutions were done

with BSS at a sample volume of 25ul. Twenty five ul of 0.5% chicken erythrocytes in BSS were used for the assay. Readings were taken after 2 hr incubation at room temperature.

Toxin-binding Assay for AchR

The method was adopted from Damle and Karline (78). To 50ul of the receptor samples which contained approximately 0.2-3.0pmol of toxin binding sites in Buffer A (100mM NaCl, 10mM MOPS, 0.2% Triton X-100 and 0.01% BSA at pH7.4) were added 7pmol of ^{125}I - α BGT in 50ul of Buffer A. The samples were allowed to incubated for 20 min at room temperature. The incubation mixtures were diluted with 2ml of Buffer B (10mM NaCl, 10mM MOPS, 0.2% Triton X-100 and 0.01% BSA at pH7.4) and transferred to prewashed DEAE-filter discs (Whatman DE-81) under low vacuum (1 psi) in a Millipore filter apparatus. The tubes containing the incubation mixtures were washed twice with 5ml of Buffer B and washings were transferred to the filters. The filters were further washed with 10ml of Buffer B and partially dried under vacuum. Radioactivity was determined by Beckman gamma counter. Control for nonspecific binding of ^{125}I - α BGT on filter discs was done similarly without the receptor.

Antibodies

About 10^4 HAU of inactivated virus particles in complete Freund adjuvant was injected subcutaneously into a rabbit. Booster injections were given at weekly intervals until a

suitable titer was obtained. Blood was taken 7-10 days after the last injection. The antiserum was incubated at 56°C for 30 min to inactivate the complement. The IgG fraction was prepared by two precipitations with 50% saturated ammonium sulfate and subsequent dialysis against BSS. Antibodies against purified AchR was a gift from Douglas Dodd. Complement inactivation and preparation of EgG fraction were performed as above.

Radioimmuno-precipitation Assay

To 50 ul of BSA (25 mg/ml), 5 ul of ^{125}I -labeled Sendai virus suspension (3 mg/ml) or AchR-enriched membrane (3 mg/ml) was added. Fifty microliters of appropriate rabbit antibody solution at different dilutions were added. Incubation was carried out for 1 hr at room temperature. Fifty microliters of goat anti-rabbit IgG was added to each tube and allowed to incubate overnight at 4°C. The precipitates were washed three times with 0.5 ml BSS by centrifugation at 3,000 rpm for 20 min. The percentages of radioactivity in the pellet were determined by gamma counting.

Similar procedure was performed for immunoprecipitation of lipid vesicles. The lipid of the vesicles was labeled with ^3H -EYL and the AchR was labeled with ^{125}I - αBGT . The free toxin and unincorporated receptor were removed by chromatography on Sepharose 4B column. Samples of 100 ul lipid vesicles were used for the assay. In the control, a final concentration of 0.7% NP-40 was used to lyse the vesicles

before the antibody was added to the mixture. Radio-immuno-precipitation assay was performed as above.

Reconstitution of AchR and AchR-VEP Vesicles

The method for reconstitution of AchR and AchR-VEP vesicles is shown in Figure 7. One ml of AchR-enriched membrane (3 mg/ml) and one ml of Sendai virus particles (3 mg/ml) were solubilized separately with sodium cholate at a final concentration of 2%. Prior to the addition of the detergent, 15 mg of the sonicated vesicles made of azolectin was added to the AchR-enriched membrane suspension. The resulting solutions were centrifuged at 100,000 x g for 1 hr to remove the insoluble membrane and nucleocapsid. The soluble AchR with azolectin was reconstituted into lipid vesicles by passing through a Sephadex G50 (fine) column (1.5 cm x 40 cm) which was equilibrated with BSS. The reconstituted vesicles were collected at the void volume. For AchR-VEP vesicles, soluble AchR and Sendai envelope proteins in a ratio of 1:1 (w/w) were allowed for co-reconstitution by the same procedure. Both kinds of vesicles were dialyzed against two changes of BSS for 24 hr before cell treatment. All procedures were performed at 4°C.

Negative Stain Electron Microscopy

A drop of membrane or vesicle suspension was deposited on a formvar coated copper grid. After 1 min, the excess sample was blotted dry and a drop of 2% sodium phosphotungstate solution buffered with 0.1M NaP at pH6.9 was

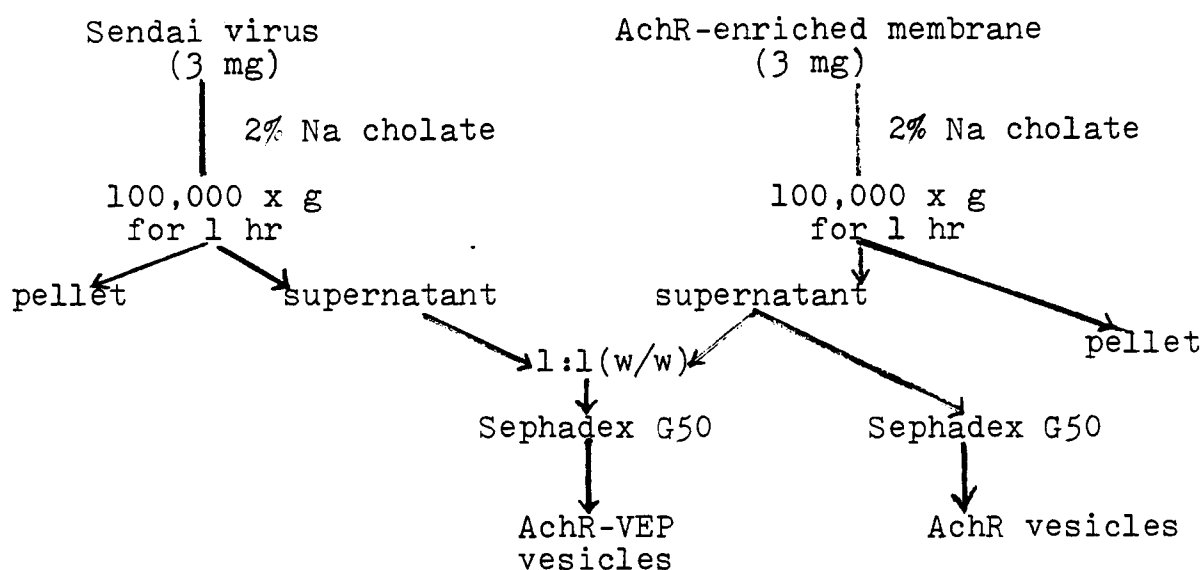


Figure 7. Method for reconstitution of AchR and AchR-VEP vesicles.

applied. After about 30 sec, the grid was dried and observed in the electron microscope operating at 60kV.

Cell Culture and Lipid-vesicle Treatment

L cells were grown on Corning plastic tissue culture plates in McCoy's 5A medium containing 10% inactivated donor calf serum at 37°C in an atmosphere of 5% CO₂ in air.

The cells were detached from the plates by incubating with 1 mM EDTA in HCMF (10mM HEPES, 138mM NaCl, 5.4mM KCl, 1.1mM Na₂HPO₄, 1.1mM KH₂PO₄ and 1 mg/ml glucose at pH7.4) at 37°C for 15 min. The detached cells were washed twice with Eagle's minimum essential medium (EMEM) without serum. About 2 x 10⁷ cells in 2 ml of EMEM were treated with 1 ml of lipid vesicles in a 15 ml Corning culture tube. The samples were rocked for 15 min at 4°C and then further incubated at 37°C for 1 hr. The cells were washed three times with EMEM and resuspended in complete culture medium.

For assaying toxin-binding sites on cell surface, the cells were incubated with ¹²⁵I-αBGT (1.8 ug) at 4°C for 1 hr with rocking. The cells were then washed six times with complete culture medium with 10% donor calf serum. Toxin-binding was estimated by radioactivity associated with cells.

For AchR turnover study, vesicle-treated cells were radiolabeled with ¹²⁵I-αBGT as above. After washing, the cells were inoculated in 60 mm plates at about 2 x 10⁵ cell/plate. At different time intervals, cells were washed three times with complete culture medium and dissolved in 0.2% SDS in NaP buffer and the radioactivity was determined.

Indirect Immunofluorescent Labeling

After vesicle treatment, cells were washed and fixed in 1% paraformaldehyde in BSS for 20 min. Then the cells were washed three times and suspended in one drop of BSS. A drop of appropriate rabbit antibody was added. The mixture was incubated at room temperature for 20 min. After the incubation, the cells were washed twice with BSS and treated with FITC-conjugated anti-rabbit IgG antibody in the same manner. The cells were washed twice and observed under the fluorescent microscope. For monolayer cell culture, similar fixation and immuno-specific labeling were performed.

Indirect Immunoferritin Electron Microscopy

After vesicle treatment, cells were fixed with 1% glutaraldehyde for 10 min and washed three times with BSS. The specific immuno-reaction was performed similarly to the immunofluorescent technique except ferritin-conjugated goat antibody against rabbit IgG was used instead of the FITC-conjugated antibody. The cells were washed and further fixed in 3% glutaraldehyde in 0.2M NaP buffer (pH7.2) at 4°C for 1 hr. After washing three times, the samples were postfixed with 2% osmium tetroxide for 2 hr at 4°C and then dehydrated in a series of aqueous acetone. Finally, the samples were embedded in Epon. Silver sections were obtained and observed with the electron microscope without further staining.

Orientation of AchR in Lipid Vesicles

Vesicle preparations were incubated in the presence or absence of 0.2% Triton X-100 with an excess of ^{125}I - αBGT for 30 min at room temperature. At the end of the incubation, a 100-fold excess of non-radiolabeled αBGT was added. The amount of ^{125}I - αBGT bound to the AchR was assayed by filter assay. The percentage of toxin binding sites exposed to the outside was calculated as follows:

$$\text{Percent of AchR exposed to the outside} = \frac{\text{^{125}I-}\alpha\text{BGT binding without Triton X-100}}{\text{^{125}I-}\alpha\text{BGT binding with Triton X-100}} \times 100\%(2)$$

Cytotoxicity Assay by ^{51}Cr Release

Monolayer L cell cultures in microtiter plates were washed three times with BSS and treated with 0.1 ml of vesicle preparations or Sendai virus for 1 hr at 37°C . After the incubation, cells were washed twice with BSS and incubated with ^{51}Cr in complete culture medium (1 uCi/well) for 1 hr at 37°C . The cells were washed twice and treated with 0.1 ml of antibody solution for 30 min at 37°C . Aliquots of 0.1 ml of freshly prepared guinea pig complement (1:20 dilution) were added in the wells and allowed for 1 hr incubation. Plates were centrifuged for 10 min at 1,500 rpm. Samples of 100 μl supernatant were taken out for measuring radioactivity. Maximal ^{51}Cr release was done by treating the cells with 1% Triton X-100 and spontaneous release was

measured for each type of treatment either without complement, antibody or vesicle treatment. The percentage of specific ^{51}Cr release was calculated as follows:

$$\begin{array}{l} \text{Percent of specific} \\ ^{51}\text{Cr release} \end{array} = \frac{E - Sp}{T - Sp} \times 100\% \quad (3)$$

while E stands for cpm released by vesicle-treated cells; Sp, for cpm by spontaneous release; and T, for cpm by maximal release with Triton X-100.

SDS-polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out following the method of Laemmli (91). The separation gel consisted of 7.5% acrylamide solution in 0.37M Tris-HCl (pH8.8) and 0.1% SDS. The gel was polymerized by the addition of 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulfate. The stacking gel contained 3% acrylamide, 0.125M Tris-HCl (pH6.8), 0.1% SDS and was polymerized as mentioned above. The ratio of acrylamide to methylenebisacrylamide was 37:1. The reservoir buffer contained 6g Tris base, 28.8g glycine and 1g SDS in 1000 ml of water. Samples from 50 to 100 ug of protein were treated with 2% SDS and 0.1% 2-mercaptoethanol and incubated in boiling water for 5 min. Electrophoresis was carried out at 3mA per gel. Gels were stained with 0.01% Coomassie Blue in 5% methanol and 7.5% glacial acetic

acid for 1 hr and destained with the same solvent system without the stain.

CHAPTER III

RESULTS

Purification of AchR-enriched Membrane

During the process of purification, a protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was included to avoid proteolysis and 0.02% NaN_3 , to inhibit bacterial growth. After the first sucrose gradient, the middle membrane band (Band II) contained more than 60% of the total toxin binding sites (Table 2). Further purification was achieved by the second gradient. The specific activity for toxin binding increased by 2.5-fold when the membrane fractions from Band II and Band V were compared. Four polypeptides with apparent mol. wt. of 40,000, 50,000, 60,000 and 65,000, which were believed to be the subunits of AchR complex, were identified by SDS-gel electrophoresis. Other impurities were present. Assuming the AchR complex had a mol. wt. of 250,000, the purified membrane fragments contained about 37% of the total protein as AchR.

Negative staining of AchR-enriched membrane fragments revealed closed vesicles (Figure 8). All of these vesicles showed rosette structures on the membrane surface. These structures were approximately 80-90Å in diameter and were similar to those reported by Nichol and Potter (22). Thus it is reasonable to consider these rosette structures as

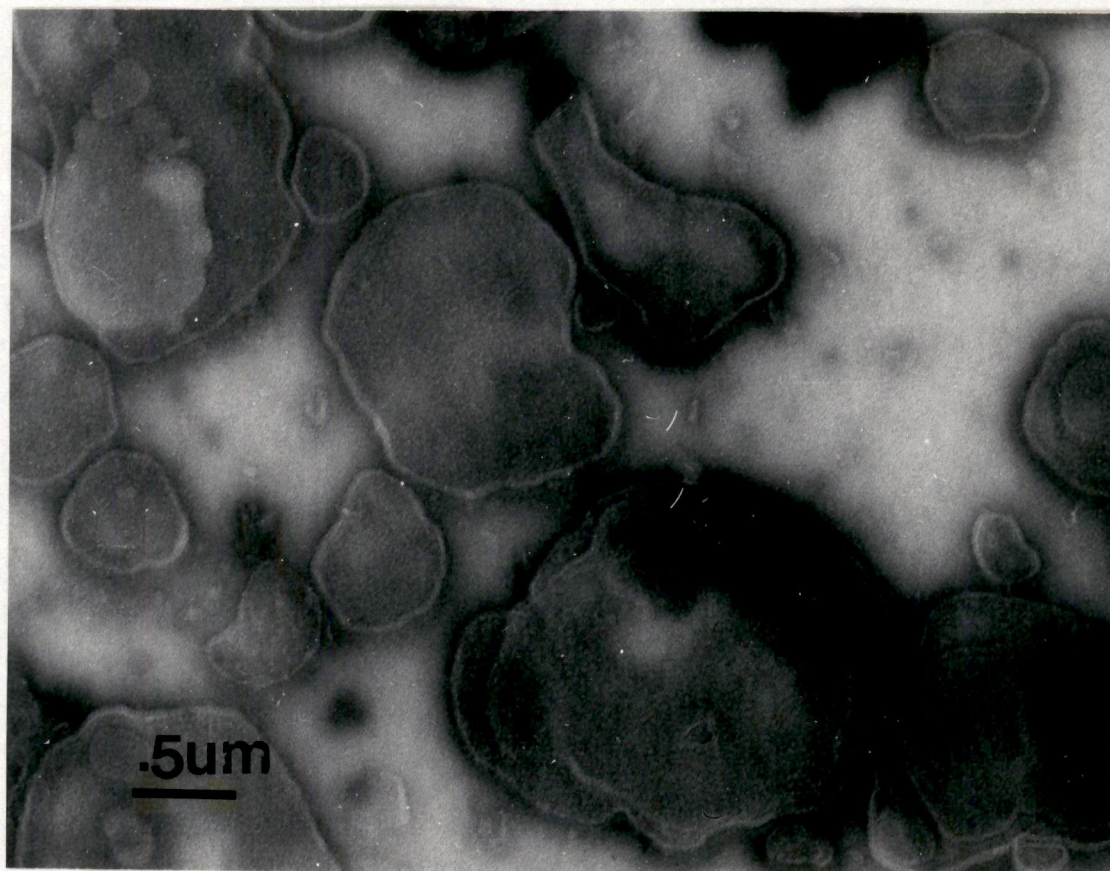


Figure 8. Negative stain electron micrograph of AchR-enriched membrane.

TABLE 2. Purification of AChR-enriched Membrane

	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Toxin binding/ protein (nmol/mg)	Total toxin binding (nmol)
Homogenate	460.0	8.4	3864.0	0.16	618.2
Pellet	30.0	11.1	333.0	0.32	106.5
Band I	8.0	4.6	36.8	0.24	8.8
Band II	12.0	11.0	132.0	0.60	87.1
Band IV	2.8	3.8	10.8	0.24	2.6
Band V	4.7	4.5	21.4	1.52	32.5

AchR molecules. The method used for isolation of AchR-enriched fragments required only differential centrifugations and sucrose density gradient centrifugations. This method provides milligram quantity of highly purified AchR-enriched membrane. These membrane fragments were shown by Reed et al. to be separated from the fragments enriched with Ach esterase and ATPase activities (23).

Purification of Sendai Virus

Sendai virus was purified by two cycles of differential centrifugation. The virus preparation was highly purified. SDS-gel electrophoresis showed all five viral proteins with little contaminations. Transmission electron microscopy showed that the virions had an average size of 0.2 μ m in diameter (Figure 9). The viral envelopes enclosing the nucleocapsids showed spike-like structures on the surface.

Preparation of AchR and AchR-VEP Vesicles

AchR-enriched membrane fragments were solubilized in 2% sodium cholate in the presence of exogenous azolectin. High speed centrifugation was performed to remove the insoluble membrane. The optimal cholate concentration for solubilizing AchR was 2%. Extraction with 1% resulted in incomplete solubilization. Reconstitution of AchR into lipid vesicles was accomplished by Sephadex G50 column chromatography. This column has a low exclusion limit and is particularly suitable for membrane protein

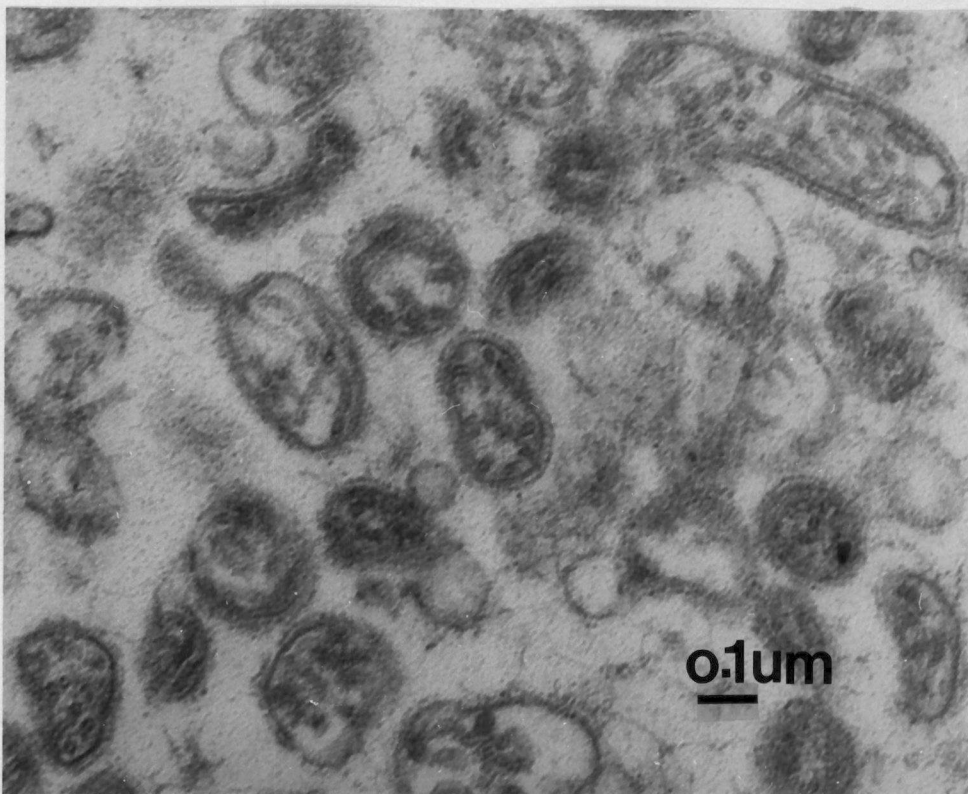


Figure 9. Transmission electron micrograph of Sendai virus.

reconstitution. Sodium cholate, which has a high critical micellar concentration, was retarded and separated from the lipid and proteins, which were eluted in the void volume as reconstituted vesicles.

When the void volume fraction from the Sephadex G50 column was chromatographed on a Sepharose 4B column to separate the vesicles and the unincorporated protein, more than 80% of the toxin-binding sites and hemagglutinating activities were co-eluted with the lipid (Figure 10). This result suggested that the AchR and the viral proteins were co-reconstituted into the lipid vesicles.

Negative stain electron micrographs of AchR and AchR-VEP vesicles are shown in Figure 11, a and b. The reconstituted AchR vesicles appeared as homogeneous vesicles with an average diameter of about 600Å (Figure 11, a). Particles were observed at the vesicle surface, which might represent the receptor molecules incorporated into the lipid bilayers. Electron micrograph of AchR-VEP vesicles showed vesicles similar size to AchR vesicles. Spikes were observed on the vesicle surface (Figure 11, b), which suggested the reconstitution of the viral envelope proteins.

The gel patterns of AchR-enriched membrane, Sendai virus, cholate extracts and the vesicle preparations are shown in Figure 12. Essentially, no difference was detected when the gel patterns of the AchR-enriched membrane, cholate extract and the AchR vesicles were compared, which indicated that complete solubilization and reconstitution of the

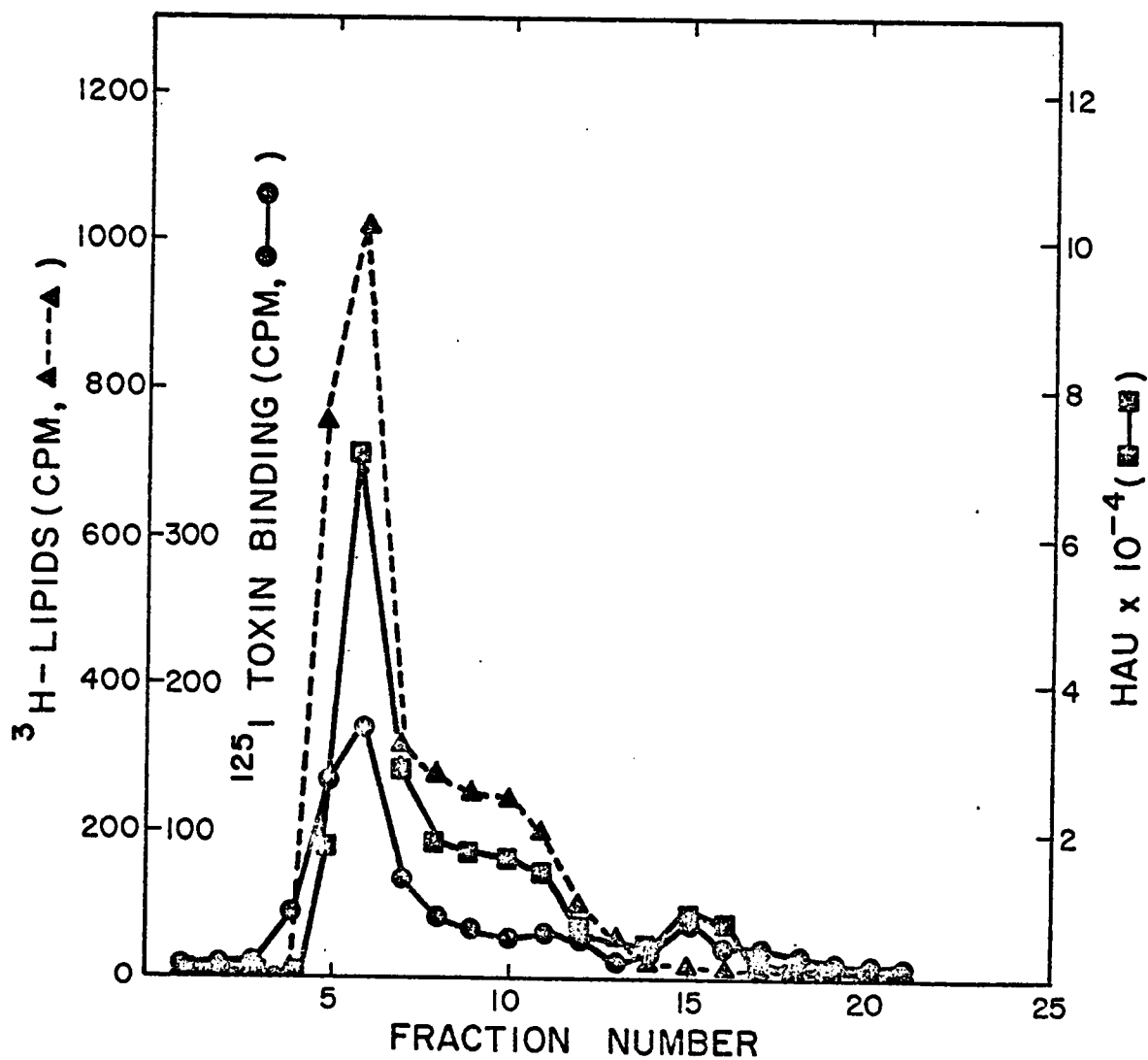
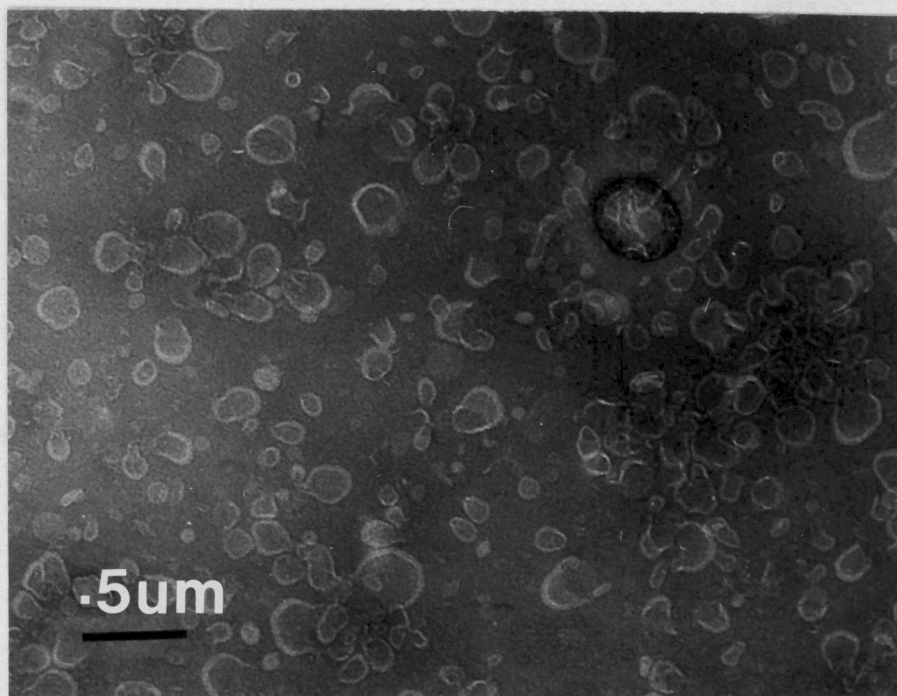
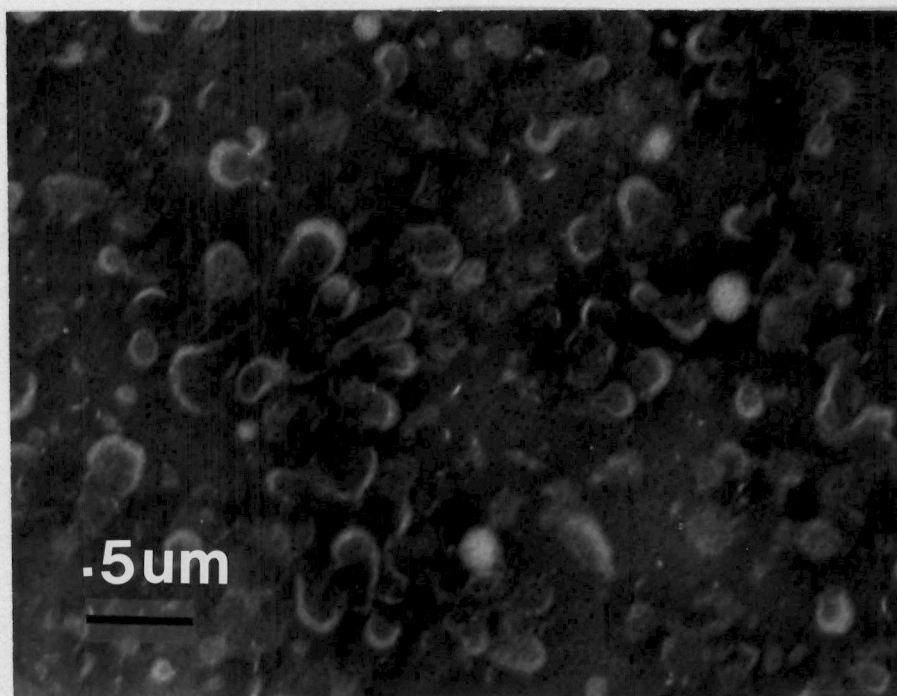


Figure 10. Elution profile of reconstituted AchR-VEP vesicles on Sepharose 4B column. Δ --- Δ , ^3H -lipid; $\bullet$ — $\bullet$, ^{125}I -BGT; $\blacksquare$ --- $\blacksquare$, hemagglutinating activity, HAU.

Figure 11. Negative stain electron micrographs of AchR and AchR-VEP vesicles. (a) AchR vesicles; (b) AchR-VEP vesicles.



(a) AchR vesicles.



(b) AchR-VEP vesicles.

Figure 11

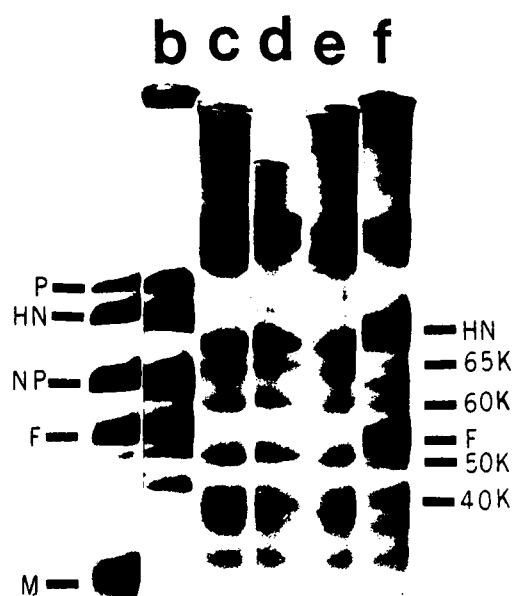


Figure 12. SDS-gel electrophoresis of Sendai virus, AchR-enriched membrane, their cholera extracts and their reconstituted vesicles. (a) Sendai virus; (b) cholera extract of Sendai virus; (c) AchR-enriched membrane; (d) cholera extract of AchR-enriched membrane; (e) AchR vesicles; (f) AchR-VEP vesicles. P stands for polymerase; HN, for HN glycoprotein; NP, for nucleocapsid protein; F, for fusion protein; M, for matrix protein; 40K, 50K, 60K and 65K, for the subunits of AchR with the corresponding molecular weights.

membrane proteins. This result was also observed by Huganir and coworkers (59). When the gel patterns of the AchR-VEP and AchR vesicles were compared, two extra protein bands which had the same mobilities as the F protein and HN protein of Sendai virus were observed. This result suggested the presence of AchR and the viral envelope proteins in the same vesicle population. Minor contamination by NP protein was observed, but no M or P proteins were detected in the gel for AchR-VEP vesicles. Throughout the reconstitution procedure, these soluble proteins were not incorporated into the vesicles.

Orientation of AchR in the Reconstituted Vesicles

The orientation of the receptors in the vesicles is of utmost importance for the transfer of this specific biological function into the cell. The proper right-side-out arrangement of the AchR on the reconstituted vesicles will result in the proper orientation in the plasma membrane after vesicle-cell fusion. The experiment was designed to examine the orientation of AchR in the vesicle populations. This could be done by testing the latency of the toxin-binding sites.

The total amount of toxin-binding sites was assayed by ^{125}I - αBGT binding after the vesicles were solubilized by Triton X-100 to release all the AchR for toxin binding. The amount of AchR exposed on the outside was determined in the following way. A saturating amount of ^{125}I - αBGT was added

to the vesicles to bind all the AchR exposed to the outside. After that, a 100-fold excess of unlabeled toxin was added before the vesicles were solubilized by detergent and subsequently assayed for radiolabeled receptor-toxin complexes. The percentage of the right-side-out AchR in the reconstituted vesicles was calculated to be 58.1 and 60.3% for the AchR and AchR-VEP vesicles, respectively. These results indicated that the receptor molecules were more or less randomly oriented during the reconstitution process.

Evidence for Co-reconstitution of AchR and Sendai Envelope Proteins into the Same Vesicle Membrane

The facilitation of AchR transfer to cell surface by Sendai viral envelope proteins demands that the AchR and the viral envelope proteins be co-reconstituted into the same vesicle membrane. Immuno-precipitation technique could be used to examine whether this was the case. The AchR molecules in AchR-VEP vesicles were labeled with ^{125}I - α BGT and the vesicle lipid with ^3H -EYL. The free ^{125}I - α BGT and the unincorporated receptors were removed by Sepharose 4B chromatography.

When the AchR-VEP vesicles were treated with anti-Sendai antibody, 64.57% of the ^{125}I - α BGT label could be precipitated, while anti-AchR antibody could precipitate 58.88% of the toxin label (Table 3). Similarly, 88.55% and 71.49% of the lipid could be precipitated by anti-Sendai and anti-AchR antibodies, respectively.

TABLE 3. Immuno-precipitation of Vesicle Components by Antibodies

	Protein			Lipid		
	Original cpm	cpm ppt	% ppt	Original cpm	cpm ppt	% ppt
R*V ¹ + anti-Sendai Ab	6924	4471	64.57	45683	40453	88.55
R*V + anti-AchR Ab	6970	4104	58.88	45683	32662	71.49
R*V + NP-40 + anti-Sendai Ab	6899	523	7.58	45683	2975	6.51
R*V + NP-40 + anti-AchR Ab	6842	4866	71.10	45683	2013	4.40
R* ² + V ³ + anti-Sendai Ab	3821	350	9.15	24645	1779	7.21
R* + V + anti-AchR Ab	3746	2553	68.15	24645	18492	75.03

¹R*V stands for AchR-VEP vesicles, R, for receptor, V, for viral proteins and * for radiolabel. Receptor was labeled with ¹²⁵I- α BGT and lipid with ³H-EYL.

²R* represents AchR vesicles with ¹²⁵I- α BGT-labeled receptor and ³H-EYL.

³V represents lipid vesicles containing only viral envelope proteins, lipid and proteins were not radiolabeled.

After these vesicles were solubilized by NP-40, 71.10% of the toxin label was precipitated by anti-AchR antibody, but only 7.58% was precipitated by anti-Sendai antibody.

In the control using a mixture of AchR vesicles, labeled with ^{125}I - α BGT and ^3H -EYL, and unlabeled viral-envelope-protein-reconstituted vesicles, the toxin and lipid labels could only be precipitated by anti-AchR antibody, and not by anti-Sendai antibody. These data strongly suggested that anti-Sendai antibody could not precipitate AchR unless they were in the same vesicle membrane as that containing the viral envelope proteins.

In all cases without detergent, the percentages of the lipid that could be precipitated were higher than those for the proteins. This may be due to the fact that antibody could easily aggregate the corresponding antigen that was in the vesicle membrane rather than those free protein molecules or small protein aggregates. It has been shown that all the reconstituted vesicles were coated with protein molecules on the surface. Thus individual vesicles would offer multiple binding sites for the antibody. Therefore, smaller quantity of the antibody was predicted to be needed to aggregate vesicles into sizes large enough to be precipitated, as compared to that required to precipitate free antigen.

The nonspecific precipitation due to trapping was less than 10% in all cases. Assuming 9% of the label precipitated was due to nonspecific precipitation, it could be

calculated that at least 50% of the AchR molecules were in the vesicles that contained Sendai envelope proteins.

Detection of Antigens on Cell Surfaces by Indirect Immunofluorescent Microscopy

The presence of AchR on the cell surface after the vesicle-cell interaction can be detected by fluorescent immunospecific labeling. Indirect immunofluorescent technique was employed in these experiments. After the vesicle treatment, the cells were labeled with appropriate rabbit antibody. A secondary FITC-conjugated goat antibody against rabbit IgG was added for visualization of antigen-antibody complexes by the fluorescent microscope. The results are shown in Figures 13, 14 and 15.

Distinct differences in the fluorescence patterns were observed on the cells after two types of vesicle treatments. Cells treated with AchR-VEP vesicles showed "ring" fluorescence after labeling with anti-AchR antibody as the primary antibody (Figure 13, a and b). Punctate fluorescence was observed after the cells had been cultured for 5 hr (Figure 13, c and d). All cells could still be labeled after 40 hr of culture, but even and diffused fluorescence was observed (Figure 13, e and f). No fluorescence was detected when AchR-VEP vesicle-treated cells were incubated with preimmune antibody (Figure 13, g and h). Similar results were obtained for AchR-VEP vesicle-treated cells when anti-Sendai antibody was used for the labeling (Figure 14).

Figure 13. Indirect immunofluorescent labeling of AchR-VEP vesicle-treated cells by anti-AchR antibody or preimmune antibody. (a through f) cells were treated with anti-AchR antibody, (g and h) cells were treated with preimmune antibody. (a, c, e and g) phase contrast; (b, d, f and h) fluorescent micrographs; (a, b, g and h) 0 hr, (c and d) 5 hr, (e and f) 40 hr after vesicle treatments.

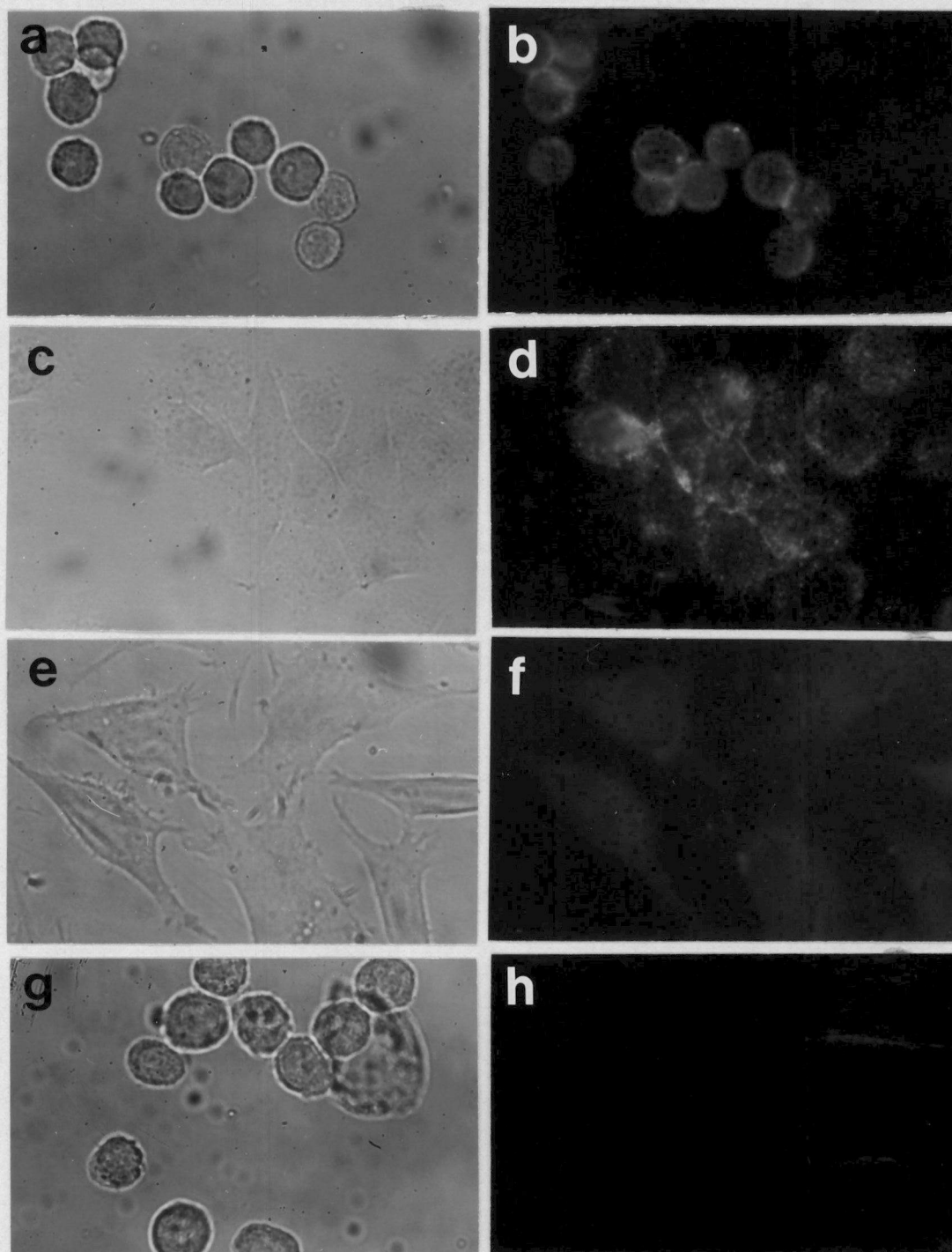


Figure 13

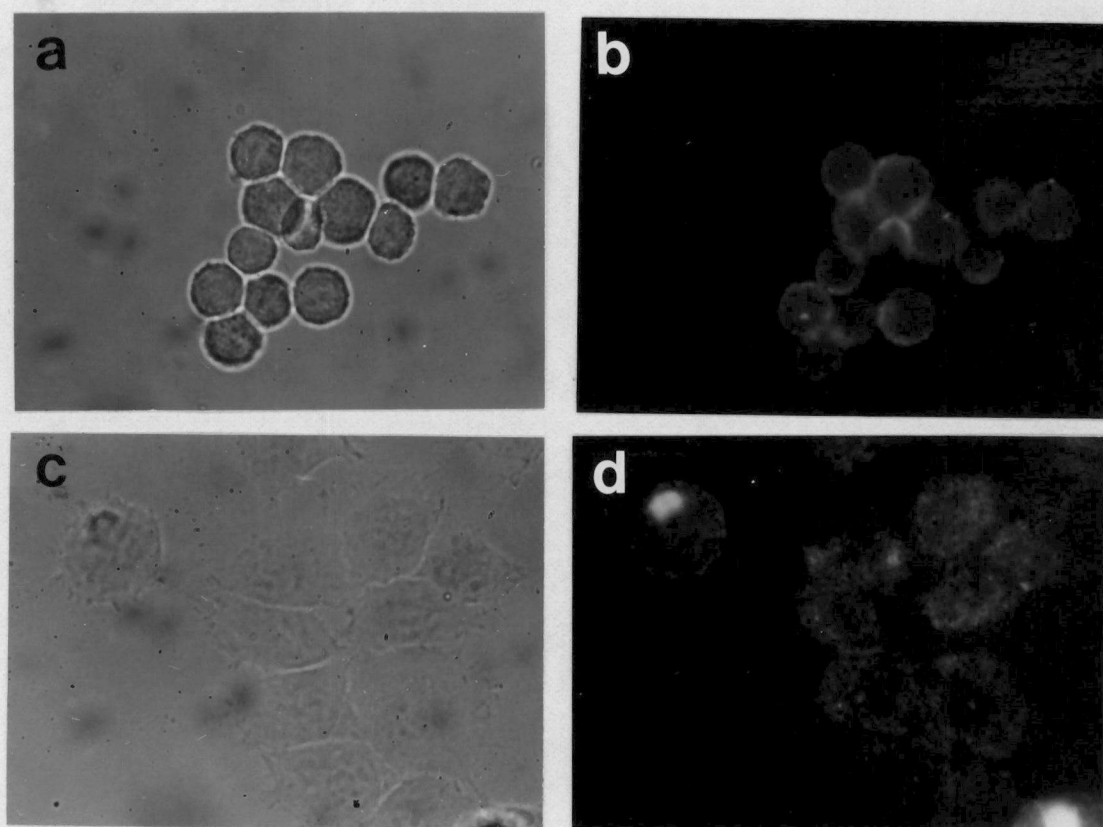


Figure 14. Indirect immunofluorescent labeling of AchR-VEP vesicle-treated cells by anti-Sendai antibody. (a and c) phase contrast, (b and d) fluorescent micrographs; (a and b) 0 hr, (c and d) 5 hr after vesicle treatments.

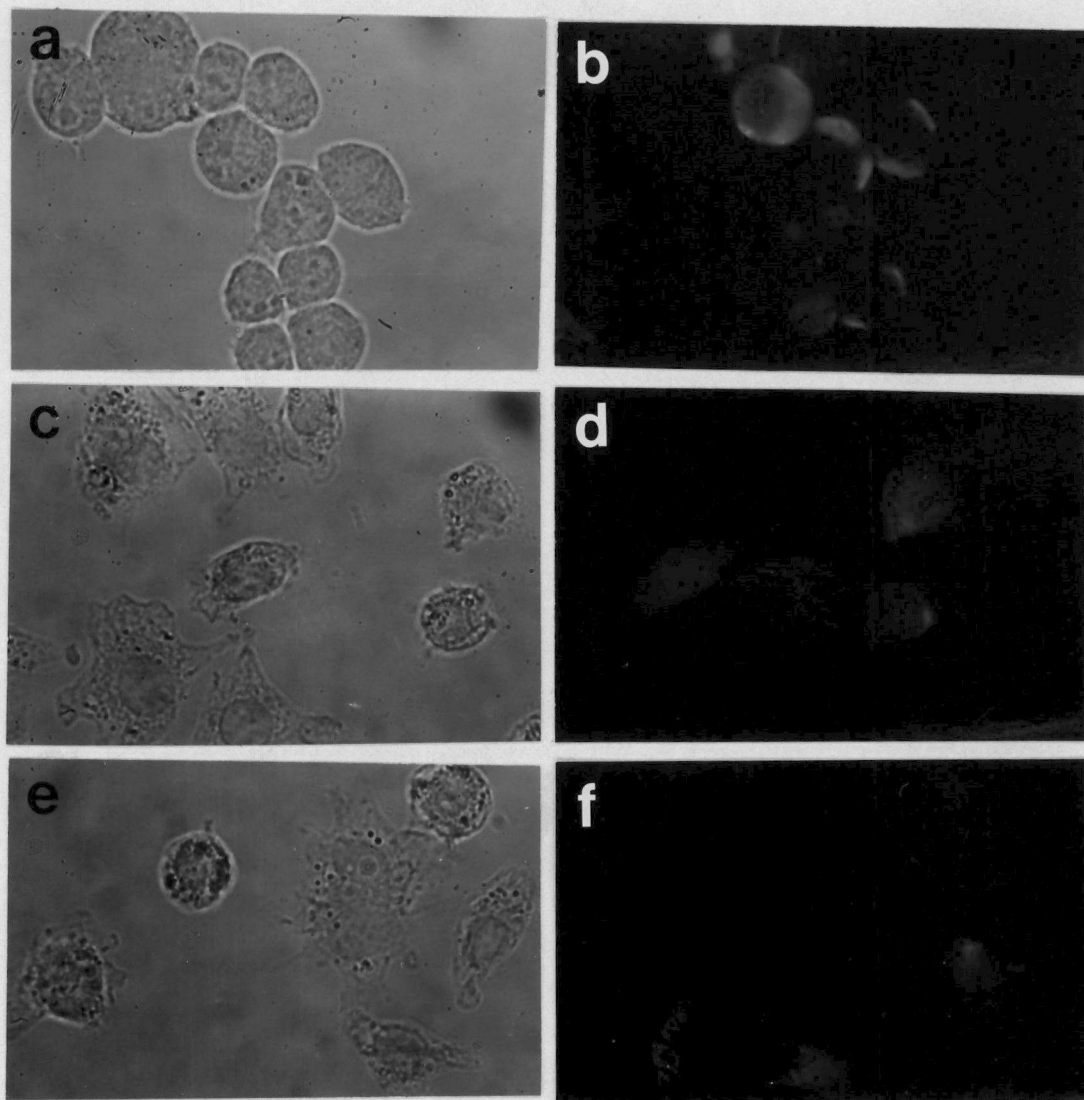


Figure 15. Indirect immunofluorescent labeling of AchR vesicle-treated cells by anti-AchR antibody. (a, c and e) phase contrast; (b, d and f) fluorescent micrographs; (a and b) 0 hr, (c and d) 5 hr, (e and f) 40 hr after vesicle treatments.

When the cells were treated with AchR vesicles and labeled with anti-AchR antibody, the cells showed patchy fluorescence on the surface (Figure 15, a and b). Not all the cells could be labeled. After further culture for 40 hr in the medium, only a small number of cells could still be labeled with fluorescent antibody and the fluorescence was mainly observed at one pole of the cells (Figure 15, e and f).

These observations suggested that AchR could be transferred onto the cell surface by the two types of vesicles. The differences in fluorescent pattern labeled on cell surfaces after different vesicle-treatments may suggest different mechanisms for vesicle-cell interaction. Further evidence to support this notion was obtained from the results of electron microscopic studies and antibody-complement fixation experiment.

Immunoferritin Electron Microscopy

AchR transferred onto the cell surface could be studied at the EM level by immunoferritin electron microscopy. This technique has high resolution and could be used to distinguish whether the AchR molecules remained in the intact vesicles or actually incorporated into the plasma membrane after the vesicle-cell fusion.

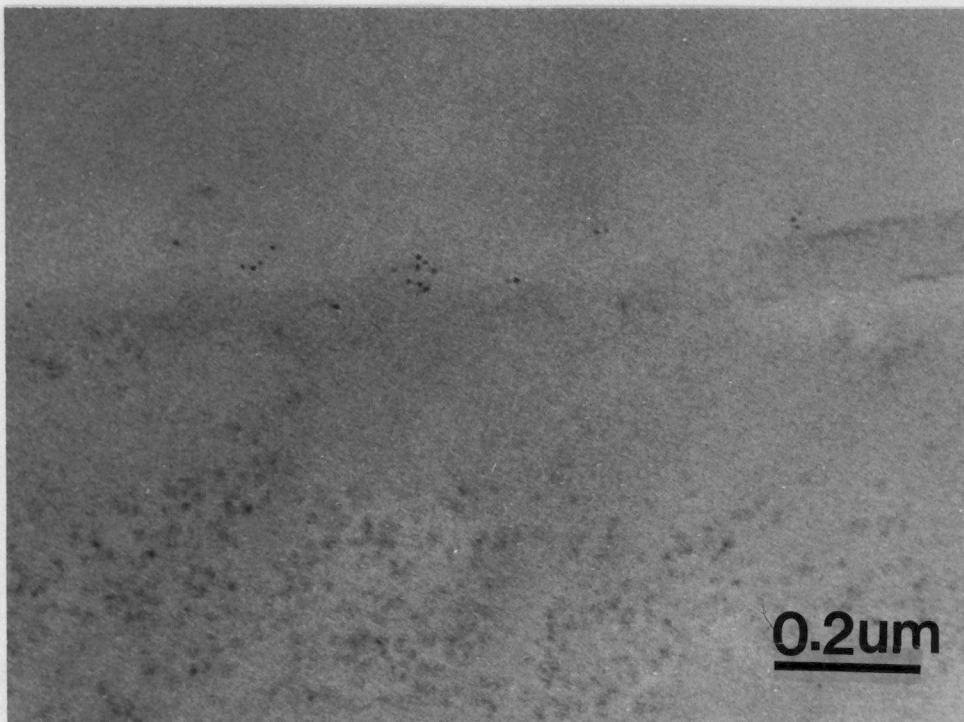
When the cells were treated with AchR-VEP vesicles and labeled by indirect immunoferritin technique with rabbit anti-AchR antibody as primary antibody, the AchR on the cell

surface could be identified by ferritin-conjugated goat antibody against rabbit IgG. The ferritin particles were identified on the plasma membrane and no vesicular structures were found associated with these ferritin particles (Figure 16, a). This observation suggested that the AchR were actually incorporated into the plasma membrane rather than adsorbed on the cell surface.

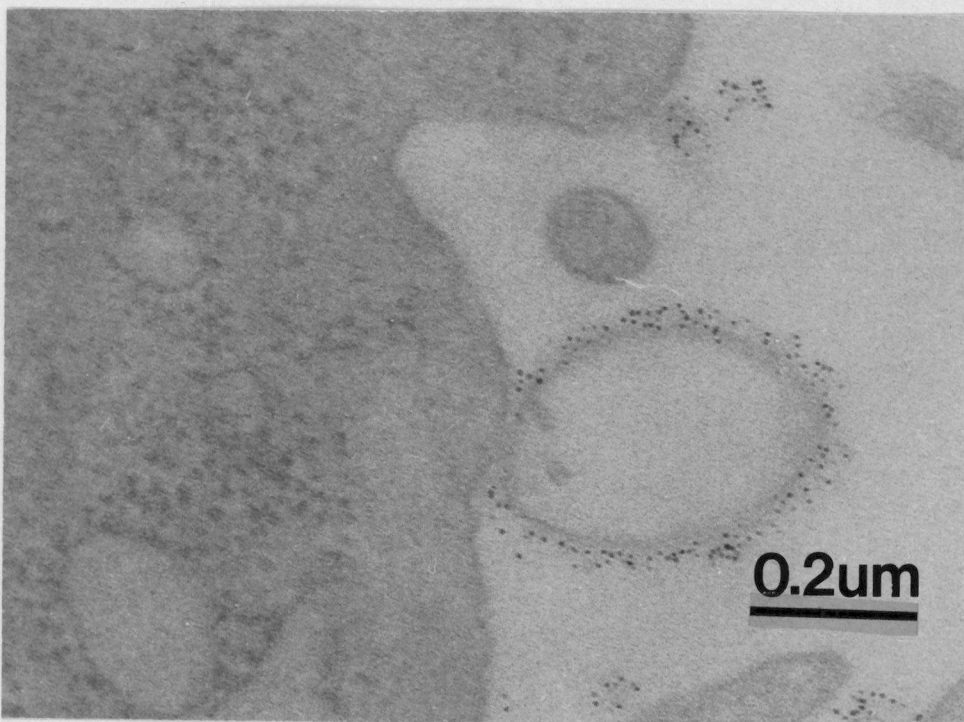
On the other hand, when the cells were treated with AchR vesicles, ferritin particles were observed at the vesicular structures located on the plasma membrane (Figure 16, b). This indicated that AchR still remained in the bilayer of the lipid vesicles without being transferred into the plasma membrane.

The electron microscopic studies again revealed the difference between the two vesicle treatments. AchR-VEP vesicle treatment resulted in the actual incorporation of the AchR molecules into the plasma membrane of the cells. When the cells were treated with AchR vesicles, vesicles were detected adsorbed on the cell surface without being integrated into the cell.

Figure 16. Indirect immunoferritin labeling of vesicle-treated cells by anti-AchR antibody. (a) Cells were treated with AchR-VEP vesicles; (b) Cells were treated with AchR vesicles.



(a) Cells were treated with AchR-VEP vesicles.



(b) Cells were treated with AchR vesicles.

Figure 16

AchR Turnover in L Cells

The usefulness of vesicle-mediated transfer of AchR into cellular membrane as an experimental tool not only demands that the introduced protein be functional, but also that this protein can persist in the plasma membrane for a sufficiently long period of time.

To investigate the turnover of AchR after being transferred on the cell surface, these receptors were labeled with ^{125}I - αBGT and the cells were cultured for different periods of time. The kinetics for the degradation of AchR was measured from the decrease of radioactivity remained on the cells. It has been shown that the dissociation of ^{125}I - αBGT from the receptors over the time period tested is negligible and the turnover of the toxin-receptor complex directly reflects the turnover of the AchR. Toxin-binding has no effect on the turnover rate of the receptor (33).

When the cells were treated with AchR vesicles, 2.8×10^4 AchR molecules per cell became cell associated, while AchR-VEP vesicle treatment resulted in 4.6×10^4 AchR molecules per cell. The turnover rates of the receptor on L cells that had been treated with AchR and AchR-VEP vesicles were compared (Figure 17). In all time periods tested, the amount of AchR remaining on the AchR-VEP vesicle-treated cells was higher than those treated with AchR vesicles. The turnover rates were rapid in both cases. For the cells treated with AchR vesicles, the half-life for

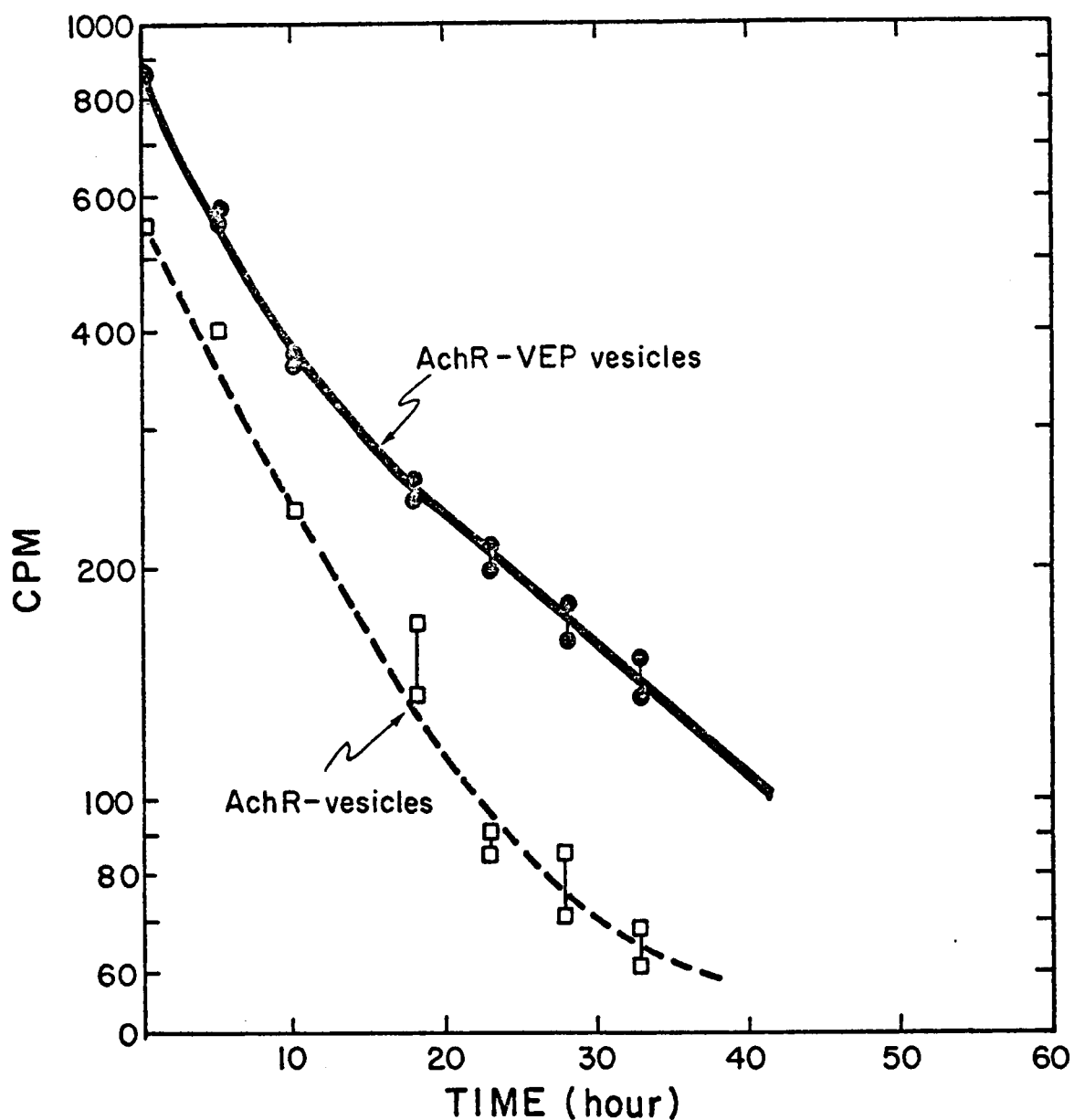


Figure 17. Degradation of AchR in L cells. Cells were treated with AchR or AchR-VEP vesicles for 15 min at 4°C and 1 hr at 37°C. The treated cells were then labeled with ^{125}I - αBGT . X-axis indicates time after vesicle treatments and toxin labeling. ●—●, toxin label remained on AchR-VEP vesicle-treated cells; □---□, toxin label remained on AchR vesicle treated cells.

the degradation of AchR was about 11.54 hr. The turnover of AchR in the AchR-VEP vesicle-treated cells was more complicated. The decay curve seemed to represent two types of turnover. About 38% of the AchR turnover has a half-life of 4.32 hr and 62% has a half-life of 23.95 hr. Endocytosis or release of the adsorbed vesicles might be the possible mechanisms for the fast turnover rate. The slow turnover might represent the actual turnover of the the receptor molecules in the plasma membrane.

Cytotoxicity Assay by ^{51}Cr Release

The reaction of specific antibody with membrane antigens of viable cells in the presence of complement may cause cell leakage and then cell death. Cell death can be determined by the release of ^{51}Cr from labeled cells. This technique is particularly useful to determine whether the AchR molecules associated with the cells are actually incorporated into the plasma membrane or not. If the AchR molecules were actually incorporated into the plasma membrane, binding with the antibody and complement could result in ^{51}Cr release. If the AchR molecules were only adsorbed on the cell surface, antibody and complement binding would not affect the cell viability and therefore no ^{51}Cr release would be detected.

Native Sendai virus particles were used as a positive control. When the cells were treated with Sendai virus, the viral envelope proteins were incorporated into the

plasma membrane of the cells. After antibody and complement fixation, there was about 13% of the ^{51}Cr released into the medium at two-fold dilution of antibody solution (Figure 18). This ^{51}Cr release was dependent on the antibody concentration. As the antibody concentration was diluted, there was a correspondent decrease of ^{51}Cr release. Similar antibody-concentration dependence was observed by AchR-VEP vesicle treatment using anti-Sendai antibody for the assay. At two-fold dilution of antibody solution, about 12% of the total labeled ^{51}Cr was released into the medium. This result could be interpreted as that after the cells were treated with AchR-VEP vesicles, the viral envelope proteins were incorporated into the cell membrane. Antibody-complement fixation caused cell lysis and specific ^{51}Cr release.

The release of ^{51}Cr by using anti-AchR antibody instead of anti-Sendai antibody was also observed (Figure 19). At two-fold dilution of antibody solution, there was about 9% of ^{51}Cr released into the medium. For AchR vesicle treatment, there was a difference of 2.87-fold when the ^{51}Cr release was compared with that for AchR-VEP vesicle treatment. Although the antibody concentration dependency for the anti-AchR antibody was less clearly observed, the 2.87-fold difference for the two samples was significant enough to suggest that AchR transferred by AchR-VEP vesicles was better than those transferred by AchR vesicles. In view of the fact that a large amount of the AchR molecules was co-reconstituted into the same vesicle membrane containing

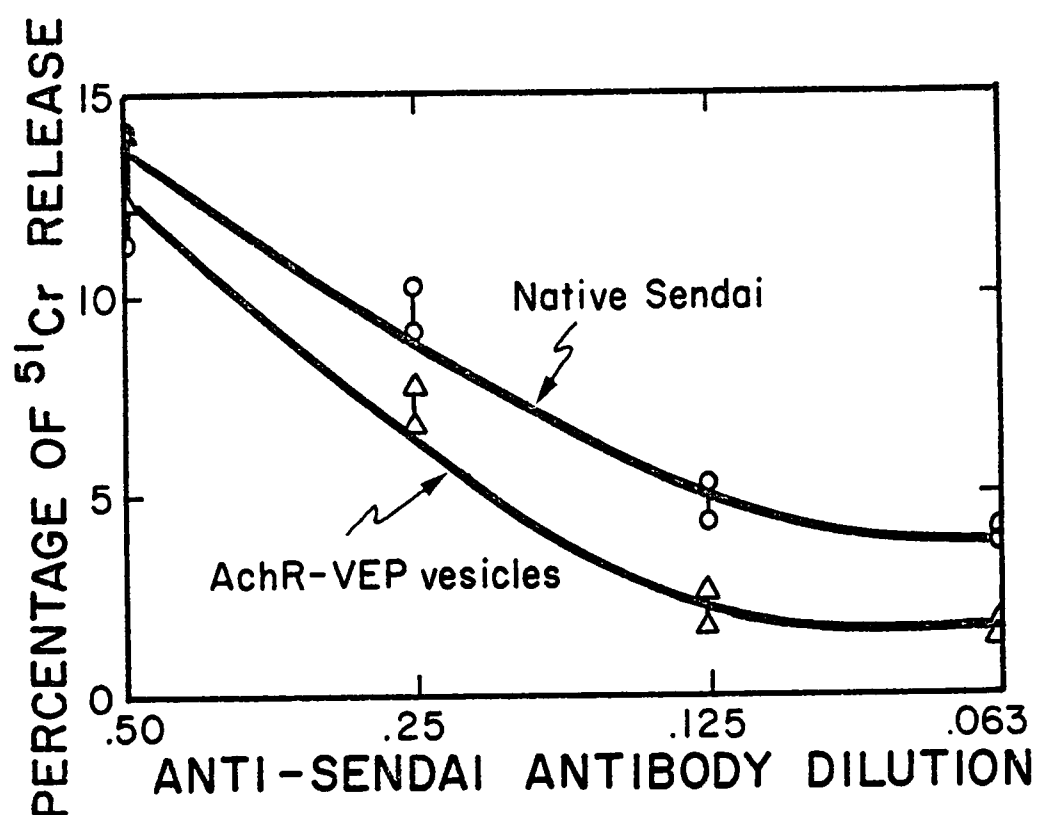


Figure 18. Antibody-complement fixation by anti-Sendai antibody. o—o, cells treated by native Sendai virus; Δ — Δ , cells treated by AchR-VEP vesicles.

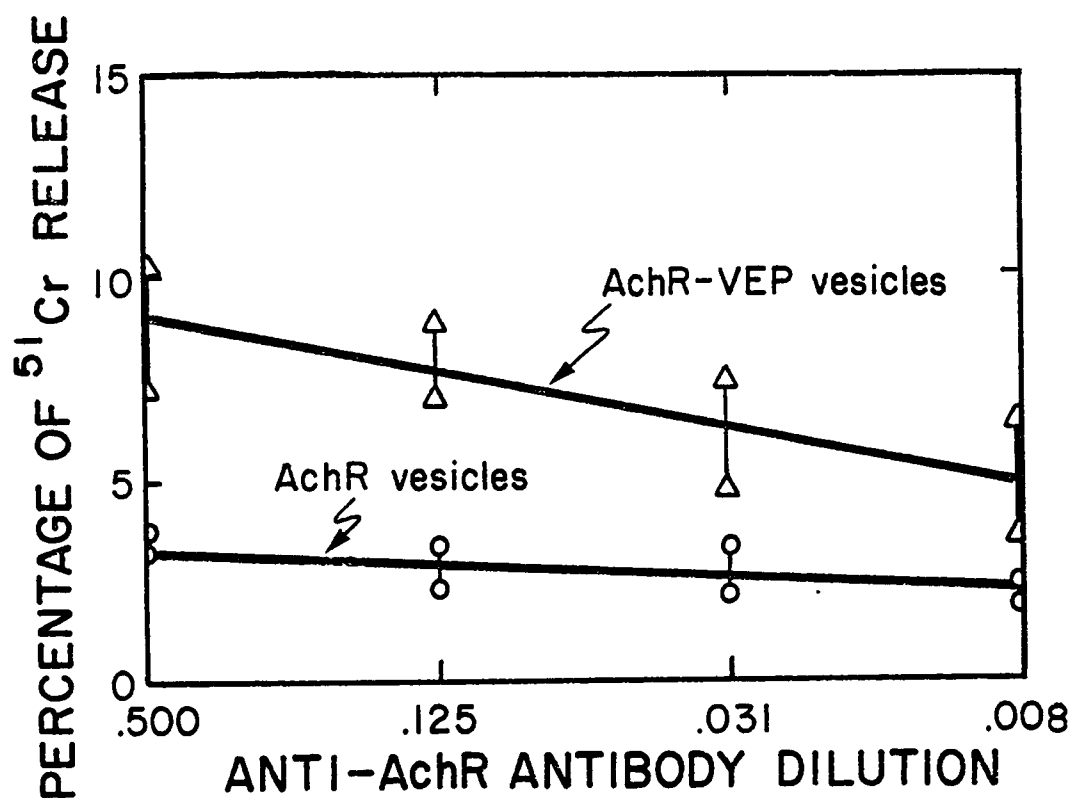


Figure 19. Antibody-complement fixation by anti-AchR antibody. Δ — Δ , cells treated with AchR-VEP vesicles; o—o, cells treated with AchR vesicles.

the viral envelope proteins, this would suggest that the insertion of the viral envelope proteins into the cell membrane would also lead to the incorporation of the AchR into the same cell membrane.

CHAPTER IV

DISCUSSION

Plasma membrane proteins are involved in a multitude of functions such as response to hormones, growth regulation, cellular adhesion, etc. A promising approach to the functional identification of any specific protein would be the isolation of the protein and reinsertion into cells lacking them. The methodology of inserting membrane proteins into cells may also provide a useful tool for modifying the plasma membrane composition and therefore the cellular functions.

The most promising experimental approach seems to be the initial reconstitution of the protein into lipid vesicles and subsequent fusion of these vesicles with cells. Upon fusion, the protein that is originally in the bilayer of the vesicle membrane becomes the integral component of the plasma membrane of the cells. In order to facilitate the fusion process, Sendai viral envelope proteins would be an excellent choice for they have the best fusion activity known. Transfer of the protein can be enhanced by the lipid vesicles containing viral envelope proteins, if it is incorporated in the same vesicle membrane as that for the viral proteins.

In the present report, a rapid technique was developed to allow co-reconstitution of AchR with the viral envelope

proteins into lipid vesicles in high efficiency. More than 80% of the AchR and viral proteins were reconstituted into the lipid vesicles. The evidences for co-reconstitution were given by co-elution of toxin-binding and hemagglutinating activities together with the lipid vesicles by Sepharose 4B chromatography, SDS-gel electrophoresis and immunoprecipitation of toxin-binding activity by antibodies against AchR or Sendai virus.

The transfer of AchR to the cell surface after vesicle-cell interactions was demonstrated by toxin-binding capacity, fluorescent antibody labeling, immunoferritin electron microscopy and cytotoxicity assay by antibody-complement fixation.

Significant amount of AchR molecules were transferred to the cells by treatment of either AchR or AchR-VEP vesicles. Although the efficiency for the AchR transfer was increased by two-fold when cells were treated by vesicles containing AchR and viral envelope proteins, distinct differences were observed after the two vesicle treatments.

When cells were treated with AchR-VEP vesicles, indirect immunofluorescent labeling showed "ring" fluorescence with either anti-AchR or anti-Sendai antibodies. Cells could still be labeled when they were cultured 40 hr after vesicle treatment and the fluorescence was rather diffuse and evenly distributed throughout the cells. In contrast, when cells were treated with AchR vesicles, irregular patches of fluorescence was observed after immunofluorescent labeling. Most

of the cells could not be labeled after 40 hr in culture and the fluorescent label in the cells was located mainly at one pole of the cell. These results suggested that the mechanisms for these two types of vesicle-cell interaction were different. Further support of this notion is given by immunoferritin electron microscopic studies. For cells treated with AchR-VEP vesicles, AchR molecules, identified by ferritin-conjugated antibody, were directly associated with cell membrane without any vesicular structures. This observation suggests that AchR molecules were most likely inserted into the plasma membrane of the cells as a result of vesicle-cell fusion. On the contrary, when cells were treated with AchR vesicles, AchR molecules were identified associated with vesicular structures adjacent to, but separated from, the plasma membrane. This suggested that AchR vesicles only adsorbed on the cell surface.

The turnover studies revealed that 62% of the AchR that were transferred by AchR-VEP vesicle treatment has a half-life of 23.95 hr. This slow turnover might represent the actual turnover of the receptor molecules in the plasma membrane. Interestingly, the half-life of these AchR molecules was similar to that for the extrajunctional receptors which also have a half-life of about 16-22 hr (32 and 33).

In mammalian muscle, there are two forms of AchR, the junctional and the extrajunctional receptors. Junctional receptors are densely packed and highly localized at motor endplates (34), while the extrajunctional receptors are found

in developing and denervated muscle at low density over the entire muscle surface. The two forms of receptors are almost identical, but can be distinguished by immunological properties. One important difference between these forms of receptors undergo continual turnover with a half-life of about 16-22 hr (32 and 33). In contrast, junctional receptors are much more stable and have an estimated half-life exceeding 6 days (32 and 34). About 62% of the AchR associated with L cells had a turnover rate similar to that of the extrajunctional receptor. This receptor preparation was originally from the postsynaptic membrane of the electric organs and therefore mainly contained the junctional receptors. These receptor molecules were observed as highly packed aggregates in the AchR-enriched membrane. After solubilization and reconstitution in the lipid vesicles, the AchR molecules were evenly distributed throughout the vesicle membrane as observed by negative stain electron microscopy. When the cells were treated with AchR-VEP vesicles, the AchR, identified by immunofluorescent labeling, were evenly distributed on the cell surface. This pattern again was similar to that for the extrajunctional receptors in the muscle cells. The observed turnover rate in the L cells might reflect the instability of the molecules if they were highly mobile and dispersed on the cell membrane.

Results from the cytotoxicity assay seems to support the idea for two types of vesicle-cell interactions.

⁵¹Cr release was observed using anti-Sendai antibody for

antibody-complement fixation when the cells were treated with AchR-VEP vesicles. The amount of ^{51}Cr release was comparable to that when cells were treated with native Sendai virus particles. The cytotoxicity was antibody-concentration dependent. When anti-AchR antibody was used for the assay, the cytotoxicity for AchR-VEP vesicle-treated cells was 2.87-fold higher than those treated with AchR vesicles. The cytotoxicity had less antibody-concentration dependency. Since the viability of the cells will only be affected by specific antibody binding to antigens incorporated into the cell membrane, the result can be interpreted that the AchR molecules were introduced into the cell membrane more efficiently by AchR-VEP vesicles than by AchR vesicles. Furthermore, most of the AchR molecules were co-reconstituted with the viral proteins into the lipid vesicles as shown by the immuno-precipitation data; therefore, it is reasonable to assume that incorporation of the Sendai viral proteins into the cell membrane as a result of vesicle-cell fusion could also lead to the incorporation of AchR molecules into the same membrane.

Although the apparent transfer of AchR to the cells was increased by two-fold when compared with the two vesicle treatments, in reality the amount of AchR actually transferred into the plasma membrane was more than two-fold. This is because a large portion of AchR was just adsorbed onto the cells when they were treated with AchR vesicles. On the other hand, most of the receptors were incorporated into

the cell membrane when the cells were treated with AchR-VEP vesicles. Besides the fact that AchR transferred by AchR-VEP vesicles to the cell surface was more efficient than that by AchR vesicles, the method is far from ideal. Assuming that the average surface area of a single L cell with a diameter of 10 μm is $314 \mu\text{m}^2$ and all the 4.6×10^4 AchR molecules associated with the cell are actually inserted into the bilayer of the plasma membrane, the calculated density for AchR is 127 AchR molecules per μm^2 , which is 7.8-fold less than that of the extrasynaptic area of a denervated muscle cell (about 1,000 AchR molecules/ μm^2) (34), and 400-fold less than that at the postsynaptic membrane (50,000 AchR molecules/ μm^2) (38). One of the ways to improve the transfer is to use purified AchR to increase the AchR density in the vesicle membrane. The reason why an impure preparation of AchR was used was to preserve the ion channel function of the receptor. This receptor is known to be highly sensitive to Ach analogue binding and detergent solubilization (47-49). Ach analogue binding in the affinity column could change the conformation of the molecules and renders them insensitive to agonist induced sodium flux. Recently, Racker et al. has purified AchR by affinity column in the presence of appropriate lipids and successfully preserved the ion channel property after reconstitution into lipid vesicles (58-60). Therefore, purified AchR can be used to increase the amount of AchR reconstituted into the vesicles and, thus, increase the receptor transfer.

The fluorescent distribution identifying the location of AchR on cells after AchR-VEP vesicle treatment is of special interest for the mechanism of synapse formation. One still knows very little of how receptors are so precisely located at the neuromuscular junctions. It has been postulated that receptors clustered together might serve as recognition sites for nerve innervation (39-40). For both embryonic development and reinnervation of denervated muscle, neuromuscular synapse formation is correlated with a high density of AchR. Thus clusters of AchR on cultured muscle cells might act as preformed postsynaptic sites. Recent studies showed that this hypothesis of preexisting sites for synaptic formation is incorrect. When the distribution of receptors was examined on a single myocyte when it was contacted by a neurite, the receptor molecules, labeled with fluorescent toxin, coalesced under the neurite, and adjacent preexisting fluorescent patches often disappeared. Neural clusters of receptor at the synapse were thus formed by recruitment of the surrounding unclustered receptors (41). Similar conclusion was drawn from the Ach sensitivity of chick myotubes in culture during innervation by embryonic chick spinal cord. New areas of high sensitivity appeared at newly formed synapses. Synapses were not observed to form at the pre-existing sensitive spots (42).

How nerves induce receptor clusters is unknown. It was hypothesised that Ach itself acted as a trophic agent,

keeping the receptors clustered at the synapse. But neuromuscular junction formed in culture in the presence of curare seems to disprove this hypothesis (43 and 44). More recent studies suggested that the physical contact of the nerve could produce a high receptor density. Later it was found that extracts of nervous tissue added to muscle cultures increase the number of AchR and increase the receptor clusters as well (45 and 46).

The transplantation of AchR to L cell culture may provide an excellent model system for the study of the molecular mechanisms of receptor clustering and synaptogenesis. Neurons and L cells do not ordinarily form synaptic junctions. The AchR incorporated on the L cell surface may be recognized by the nerve tissue when they come in contact and synaptic junction will probably be formed. The molecular events of how clusters of AchR can be induced can be followed by fluorescent-labeled toxin. The mechanism of AchR clustering is of particular significance because clustering of receptors in the neuromuscular contact is one of the early events in synapse formation.

The functional identity of the individual subunits of the receptor may also be worthwhile to pursue. Furthermore, the incorporation of AchR into the L cell membrane could alter the cellular function. The ways of how these modified cells responded to a coming neuron remain a mystery.

The ability of using Sendai viral envelope proteins to mediate the transfer of specific protein has to be

recognized. These viral proteins have a broad specificity. Essentially, they can be used for almost any cell type. Moreover, they are very stable and do not have viral infectivity and cell-cell fusion activity that would complicate the system, but they retain their ability to fuse with cells after reconstituted into lipid vesicles. Therefore, these reconstituted viral envelopes can be used to transfer membrane proteins in cell membrane.

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