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CHARACTERIZATION OF LARGE UNILAMELLAR PHOSPHOLIPID VESICLES AND THEIR INTERACTIONS WITH CULTURED CHINESE HAMSTER CELLS

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

The present study is to find method(s) of preparing large unilamellar vesicles (LUV) for delivery of biological active materials into cells for therapeutic applications. LUV composed of egg yolk lecithin (EYL) were prepared by lipid-ether injection method of Deamer and Bangham (24). They were characterized in terms of structure, size, trapping efficiency, and permeability to small nonelectrolytes. These LUV were unilamellar of the average diameter 0.2 to 0.4 μm. The trapping efficiency determined by using ^{3}H-methoxy-inulin as an entrapped marker was 10.2 ± 1.3μl/μmol of lipid. The permeability behavior of these vesicles to nonelectrolytes was similar to that of the multilamellar vesicles.

The interactions of Chinese hamster V79 cells with LUV were examined in vitro. A minor proportion of EYL uptake from the vesicles was low-temperature sensitive and energy dependent. However, the rate of lipid uptake by the cells was found to be greater than that of trapped content uptake, suggesting that most vesicles were not taken up as intact structures. This result eleminates adsorption, endocytosis, non-leaky fusion as the major pathway for vesicle uptake. The differential rates of uptake of the vesicle membrane components argues against leaky fusion as the major process. Therefore, the mechanism of lipid transfer is inferred as the major pathway for phospholipid uptake.

Electron microscopic studies of cells incubated with LUV revealed membranous material in cytoplasmic vacuoles, resembling secondary lysosomes. Cells treated with LUV entrapped with 5-bromo-4-chloro-3-indolyl phosphate, a histochemical substrate for lysosomal acid phosphatase,

showed blue granules appeared in the cytoplasm of the cells. These results indicate that some LUV are endocytosed by the cells. Cells treated with vesicles containing a fluorescent dye, 6-carboxyfluorescein (6-CF), showed a diffused, uniform distribution of fluorescence throughout the cells, even in the presence of endocytosis inhibitors. These observations were interpreted as the release of the entrapped 6-CF into the interior of the cells after vesicle-cell fusion.

It is concluded that LUV can be employed as a carrier to deliver biologically active substances into various subcellular compartments, and thus are potentially useful as a therapeutic vector.

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CHAPTER I

INTRODUCTION

A. LIPID VESICLES

Lipid vesicles, or lipososmes, have been for a long time a model system for the study of biological membranes. They are now emerging as vectors for delivery of drugs, enzymes and hormones for therapeutic purposes. This brings lipid vesicles from a theoretical field of a membrane model into a field of practical medicine.

Multilamellar Vesicles (MLV) and Small Unilamellar Vesicles (SUV)

Two major kinds of lipid vesicle have been extensively studied: multilamellar vesicles (MLV) and small unilamellar vesicles (SUV). MLV can be prepared by hydrating and swelling phospholipids in aqueous solution (1). These vesicles are composed of a large number of closed concentric phospholipid bilayers. They are of different size up to 10 μm in diameter. During the formation of lipid vesicles, aqueous medium is entrapped between the lipid bilayers, where it is isolated from the external aqueous environment by the closed bilayers. A solute will be entrapped when it is dissolved in the aqueous solution. The untrapped solute can be separated from the vesicles by gel filtration or by repeated centrifugation and washing. Ultrafiltration can be used to concentrate the vesicles after gel filtration.

SUV are formed when MLV are subjected to ultrasonic irradiation prior to gel filtration or centrifugation (2,3). This breaks them into smaller vesicles of more homogeneous size (250-500Å). Two other methods

described recently do not require sonication. One method involved injection of ethanolic solution of phospholipid into a large volume of aqueous buffer (4). The other involves solubilization of phopholipid in detergent with subsequent slow removal of the detergent by dialysis (5,6). All these three methods result in vesicles composed of a single lipid bilayer.

Uptake of Lipid Vesicles in vivo

Lipid vesicles were first considered as possible therapeutic carriers of amyloglucosidase in the treatment of glycogen-storage diseases by Gregoriadis, Leathwood and Ryman (7). Amyloglucosidase has been successfully trapped in MLV. When they were administered intravenously 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. These results suggest that this method might be useful in treatment of storage diseases, where the biochemical lesion is normally the absence of a single enzyme.

This method can also be used as a kind of cancer chemotherapy by delivery of the entrapped cytotoxic agents to the malignant tissues. Vesicle-entrapped actinomycin D (8,9) and cytarabine hydrochloride (10) administered to tumor-bearing mice were found superior to the free drugs in prolonging the survival of such mice. This effect was attributed both to the increased uptake of the entrapped drugs by tumor cells, of which endocytotic activity is elevated, and to the reduction in the

general toxicity.

Rahman et al. have also used MLV as carriers for introducing chelating agents into cells *in vivo* (11,12). The entrapped polyaminopolycarboxylic acids EDTA was used therapeutically in the treatment of heavy-metal poisoning in mice that had intracellular deposits of toxic metals, such as plutonium, lead and inorganic mercury.

The entrapment of therapeutic agents or other biologically active materials within vesicles for delivery to cells *in vivo* offers a number of advantages over the straight forward administration of the free drug. Entrapment of materials within the immunologically inert lipid vesicles avoids provoking the host immune response. It also protects them against rapid breakdown by enzymes present in the circulation and various tissue fluids.

Intravenously injected lipid vesicles are preferentially taken up by liver and spleen. While these findings are useful to direct lipid vesicles and their contents to these tissues, some means of blocking this uptake and means of directing the vesicles to other tissues must be found. Attempts to saturate the liver uptake with diethylpalmitate in Tween 20 appears to block hepatic uptake (13). On the other hand, direction of lipid vesicles to tissues other than liver and spleen, by alteration of lipid composition has so far been unsuccessful *in vivo*. However, addition of antibodies to the vesicle surface increases the rate of uptake of these vesicles by cultured cells *in vitro* (14). Immunoglobulins have been shown by Weissmann and his collaborators to bind to lipid vesicles and to influence their uptake into cells in culture. The effect of these immunoglobulins *in vivo* is not known. Preliminary data suggest that several

serum proteins may influence vesicle uptake (15,16).

Uptake of Lipid Vesicles in Vitro

The potential of lipid vesicles as carriers to deliver materials into cells is well recognised as shown by the results in the in vivo studies. Problems are raised when the fate of vesicles mainly end up in the lysosome where the entrapped materials may be digested away by the lysosmal enzymes. Techniques have been developed to study the mechanisms of vesicle-cell interaction in vitro in order to find other pathways of delivering materials into cells, and to find out the factors that govern different pathways of uptake so as to optimize the conditions suitable for different types of delivery.

Evidence to show SUV are actually incorporated to the cell is provided by the recovery of vesicle components from subcellular fractions (17,18) and by the demonstration of vesicle components within the cells by electron histochemical technique (17) and electron autoradiography (18). Also the entrapped materials after getting inside the cells can also produce specific changes in cellular metabolism and cell behavior (19). These results provides convincing evidence that vesicles are actually incorporated into cells, not merely adsorbed to the cell surface.

It has been suggested (20,21) that the mechanism by which vesicles interact with cells is dictated to a large extent by the identity of lipids in both lipid vesicles and cell membranes. "Solid" vesicles at temperature below the liquid-crystalline phase transition temperature (Tc) are incorporated into cells primarily by endocytosis or adsorption. Vesicles that are taken up by fusion have to be in the fluid state. In

order to direct vesicles to specific target tissues, the lipid composition of vesicle membrane can be modified in terms of charge and fluidity, or by incorporation onto the vesicle surface of cytophilic molecules, such as anti-cells IgG immunoglobulins or glycoproteins that are recognized by target cells.

Large Unilamellar Vesicles (LUV)

Most of the studies have been focused on the use of MLV and SUV. Both of these vesicles have certain drawbacks. In MLV, a major fraction of the lipid is present in the multilamellar form and leaves only a small amount of aqueous space, therefore, the trapping efficiency is low. Furthermore, most of the entrapped materials cannot be released to exert their effects even after getting into the cell unless the lamellae are broken down. The SUV are very small and the trapping efficiency is too low to be practical.

Large unilamellar vesicles have recently been prepared by Papahadjopoulos (22) by fusion of SUV in the presence of Ca^{2+}. This method is limited for phosphatidylserine vesicles only.

Deamer and Bangham (24) have introduced an ether-injection method, and it works for a wide variety of lipids. In the present study, LUV of egg yolk lecithin (EYL) are prepared by this method and are examined in terms of their structure, size, trapping efficiency, permeability to small molecules and finally, their interaction with Chinese hamster V79 cells.

CHAPTER II

METHODS AND MATERIALS

A. LIPIDS

Egg yolk lecithin (EYL) was isolated from chicken egg yolks, and purified by chromatography on silicic acid. ^{14}C-dioleoyl lecithin (DOL) (Applied Science Labs. Inc.), cholesterol (Sigma) were purchased from commercial sources and used without purification. ^{3}H-dipalmitoyl lecithin (DPL) was prepared by a catalytic hydrogenation with tritium gas of dipalmitoleoyl lecithin (New England Nuclear) and subsequently purified by silicic acid column chromatography. EYL was labeled by adding a trace amount of ^{3}H-DPL or ^{14}C-DOL. The purity of lipids was examined by thin layer chromatography.

B. PREPARATION OF LARGE UNILAMELLAR VESICLES (LUV)

The procedure of making large unilamellar vesicles was essentially the method of Deamer and Bangham (24). Lipid-ether solution of definite lipid concentration (1.6mg/ml) was injected into an aqueous solution at a temperature higher than the boiling point of ether. As ether came in contact with the aqueous solution, it immediately evaporated. The lipid left in the aqueous solution spontaneously formed vesicles.

The apparatus is shown in Figure 1. A glass syringe contained the lipid-ether solution. This solution was injected into the aqueous solution through a fine polyethylene tubing (I.D. 0.032 in.). The

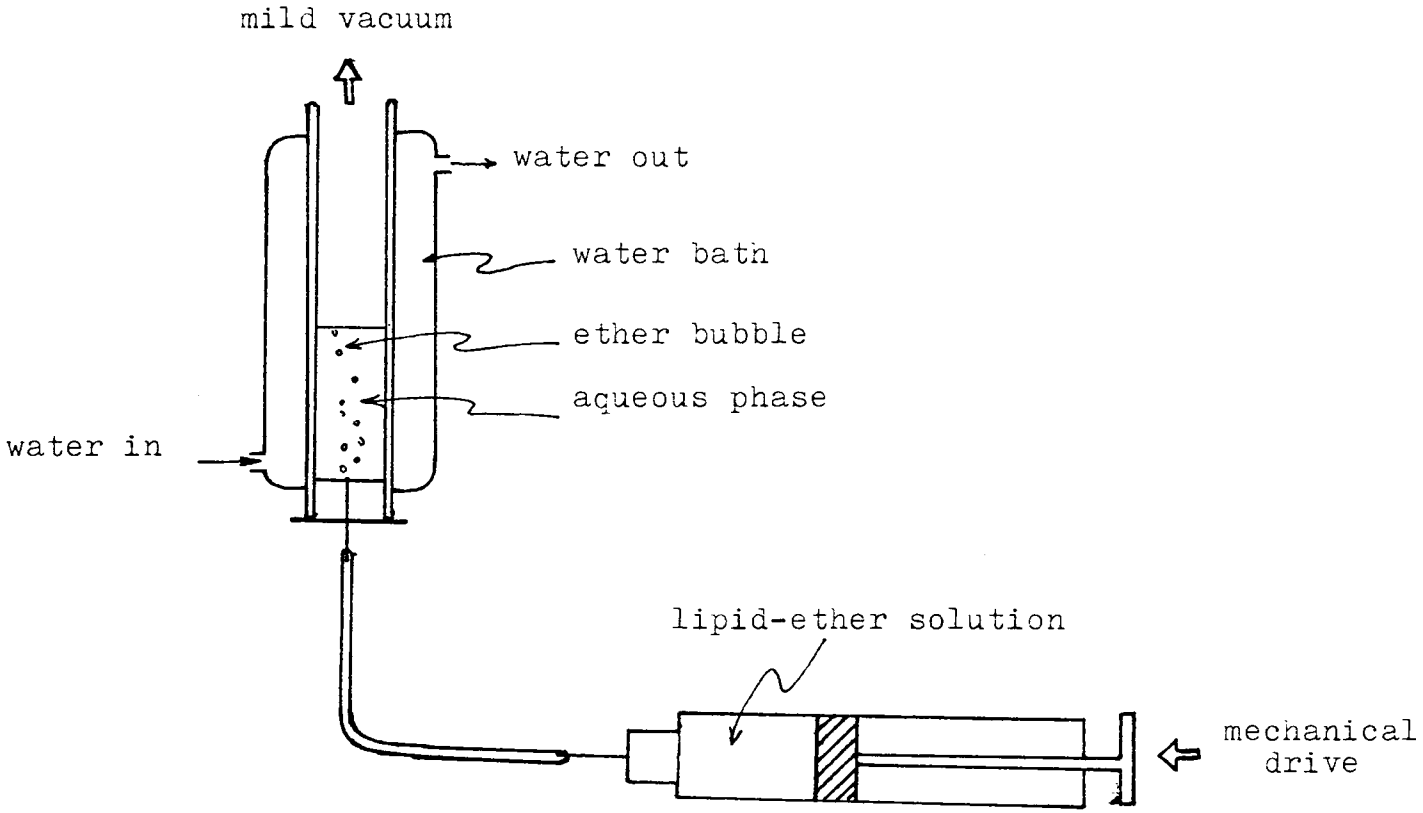


Figure 1. Diagram of the apparatus used to prepare LUV

temperature of the aqueous solution was controlled by a water jacket. An infusion pump was used to maintain a constant rate of injection. A mild vacuum applied to the aqueous solution facilitated the removal of ether vapor.

The conditions used for making LUV are listed in Table I, except otherwise mentioned.

TABLE I

OPTIMAL CONDITIONS FOR MAKING LUV

EYL concentration in ether	2.0 μmol/ml
Rate of injection	0.2 ml/min
Temperature of aqueous solution	55°C
Final concentration of lipid in the aqueous phase after lipid-ether injection	2.0 μmol/ml

After the injection, the vesicle suspension was washed three times by centrifugation at 3,000 x g for 20 min to remove the residual ether and some vesicles too small to be pelleted. If certain solutes were to be entrapped in the LUV, the untrapped molecules would be washed away by centrifugation. The resuspended vesicles were filtered through a 1.2 μm millipore filter to remove any extra large or clumped vesicles.

The vesicle suspension was then adjusted to the desired concentration.

C. ELECTRON MICROSCOPY

Thin Sectioning and Negative Staining of LUV Preparation

The method of Miyamoto and Stoechenius (25) was followed. Approximately 1.5 mg of vesicles in suspension was added to 3 ml of 2% OsO_4 at 4°C and kept in cold for 10 hr. Five volumes of 0.5% gelatin solution were added to prevent close packing. 0.1 ml of this mixture was applied on a millipore filter (0.22 μm pore diameter) under gentle suction. The filter was then dehydrated in a graded series of ethanol followed by immersion in propylene oxide which dissolved the filter. The resulting gelatin film was infiltrated and embedded in Epon. Thin silvery sections were obtained and stained with uranyl acetate and lead citrate. The sections were examined in JEM electron microscope at 80 kV.

Vesicles were negatively stained with 1% phosphotungstic acid at pH 7.2 on a carbon coated grid.

Transmission Electron Microscopy for V79 Cells Treated with LUV

Washed cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2, followed by post fixation in 1% OsO_4, dehydration in graded series of acetone, and embedding in Epon. Thin sections were stained with uranyl acetate and lead citrate.

D. DETERMINATION OF TRAPPING EFFICIENCY

^{3}H-labeled methoxy-inulin was used as a marker for the trapped content in the vesicles. Trace amount of ^{14}C-DOL was used to label the EYL lipid component. After the formation of vesicles, the untrapped

radioactive marker was washed away with Hepes calcium-magnesium-free saline (HCMF) by centrifugation. Leakage of entrapped methoxy-inulin during washing was insignificant. After millipore filtration, radioactivities in LUV were determined. Assuming the concentration of ^{3}H-labeled methoxy-inulin trapped in the vesicles was the same as that in the original aqueous solution, the trapped volume per unit mass of lipid was calculated. The trapping efficiency is expressed in μl/μmol of lipid.

E. PERMEABILITY OF LUV TO NONELECTROLYTES

The method of Hill and Cohen was followed (26). Lipid vesicles swell and shrink under the action of osmotic pressure. Volume changes, induced osmotically can be followed by the change in light scattering at 450 nm. Empirically, Bangham and coworkers found the absorbance at 450 nm is inversely proportional to the volume of vesicles (33). The experimental conditions were chosen such that the lipid vesicles first shrink under an induced osmotic force, pass through a minimum volume and then swell as permeant on the outside diffuses in across a concentration gradient, followed by its concomitant water, until equilibrium is reached. The maximum rate of volume change after passing through a minimum volume is related to the permeability coefficienct (ω) and the reflection coefficient (σ) as represented in the following equation.

$$1/dVa/dt = \frac{Ci}{Cs} \cdot \frac{1}{S\omega\sigma RT} + \frac{1}{S\omega RT}$$

Va is the volume of the aqueous compartment of the lipid vesicle; Cs is the outside concentration of the permeable species; Ci, of the impermeable species. R is the gas constant and T, the absolute temperature, and S, the surface area of the vesicle. If the change in absorbance is small, dVa/dt is directly proportional to dA/dt, the rate of change in absorbance at 450 nm. A straight line in a plot of 1/dA/dt vs Ci/Cs indicates the ideal osmotic behavior of LUV. The intercept at Ci/Cs = 0 is proportional to the reciprocal of the permeability coefficient ($1/\omega$) and the slope divided by the intercept is the measure of the reciprocal of the reflection coefficient ($1/\sigma$).

In this experiment, the nonelectrolytes, erythritol, malonamide and urea were used as permeable solutes and KCl, as impermeable solute. LUV of 1:1 molar ratio of EYL and cholesterol were made in 20 mM KCl and 0.18 ml of this was rapidly added to 2.5 ml of various hypertonic solutions of KCl and nonelectrolytes mixed in the following concentration ratios (mM): 0:100, 5:100, 10:100, 20:100, 20:60, 20:40. The final lipid concentration was 0.5 mM. Volume change was followed by the change in absorbance at 450 nm. The time courses of the optical changes were monitored by a recorder. The linear portion of the optical changes after minimum volume was used to obtain the slope, i.e. dA/dt = constant and the slope was taken in that range. The values of the reciprocal of the change in absorbance were plotted against the ratio of Ci/Cs.

F. CELL CULTURE AND VESICLE UPTAKE

Cell Culture

The Chinese hamster V79 lung fibroblast cell line (27) was used

in this study. Cells were grown on Falcon plastic tissue culture plates in Eagle's minimal essential medium supplemented with 2% lactose and 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 in air.

Preparation of Single Cell Suspensions

Cell monolayer which had just attained a confluent state were used for preparing suspensions of single cells. Monolayer cultures were rinsed three times with PucK's Ca^{2+} and Mg^{2+}-free saline (CMF) (32), and incubated (10 ml per plate) with 1 mM EDTA in HCMF for 15 min at 37°C. The detached cells were washed twice in HCMF, and finally suspended in HCMF.

Vesicle Uptake

Freshly prepared cell suspensions (2 x 10^6 cells/ml) were incubated in HCMF with radiolabeled vesicles (1.25 μmol/ml) for different time intervals. Incubations were terminated by washing the cells three times with HCMF. The final cell pellet was solubilized in 1% triton X-100 and radioactivity was counted in a liquid scintillation counter with triton-toluene fluor. Duplicate samples were made and the variation was within 5%.

Fluorescence Microscopy

The 6-carboxyfluorescein (6-CF)-containing LUV were prepared in 200 mM 6-CF in HCMF. The vesicle suspension was washed free of untrapped dye with HCMF by centrifugation. Cells were then incubated with 6-CF containing vesicles. In this case, 5 μmol of vesicle lipid was applied to 2 x 10^6 cells in 1 ml. After 2 hr of incubation, cells were washed

three times in HCMF, and examined in a fluorescence microscope.

Histochemical Demonstration of Endocytosis

The histochemical method to demonstrate endocytotic activity is based on the localization of the end product of the vesicle-entrapped marker after lysosomal digestion in the secondary lysosomes.

5-Br,4-Cl,3-indolyl phosphate (BCIP), a colorless and soluble compound, is appropriate for this study because it can readily be hyrolysed by acid phosphatase in lysosomes. After enzymatic hydrolysis 5-Be,4-Cl,3-indoxyl is formed. It is unstable and is readily transformed to the blue colored precipitate of 5,5'-dibromo,4,4'-dichloro-indigo. This can be readily seen in phase-contrast light microscope at the sites of enzymatic activity within the cells. The enzymatic reaction of this compound is shown in Figure 2.

In the experiment, BCIP-containing vesicles were prepared in the presence of 2 mg of this compound per ml of HCMF. Cells were incubated with these vesicles for 1 hr at 37°C. After washing, the cells were examined in a light microscope.

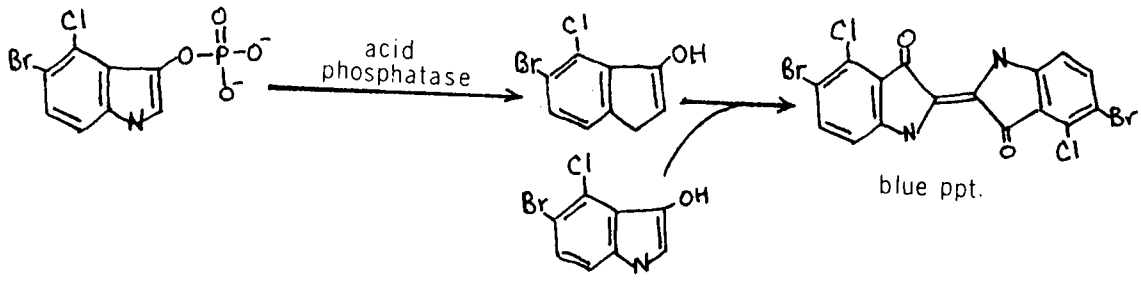


Figure 2. The enzymatic digestion of 5-Br,4-Cl,3-indolyl phosphate by acid phosphatase

CHAPTER III

CHARACTERIZATION OF LARGE UNILAMELLAR VESICLES

Figure 3 shows the electron micrograph of LUV before millipore filtration. This preparation consisted of vesicles of different sizes. Some of the vesicles were as large as 2 μm in diameter. Vesicles with more than one layer of membranes were occasionally seen. They showed a vesicle-within-vesicle structure which were different from the "onion" structure of MLV. Figure 4 shows the electron micrograph of these vesicles after millipore filtration. Note that the filtered preparation consisted of vesicles more homogeneous in size. The size distribution of these vesicles is shown in Figure 5. Most of the vesicles were of 0.2 to 0.4 μm in diameter as determined by direct measurement from the micrograph. The size determined in this way may deviate from the actual size because of the vesicles appeared to be collapsed after they were dehydrated for EM processing.

Thin sections of these vesicles shows that a large proportion of these vesicles were unilamellar (Figure 6). Some of the distorted vesicles seen in this electron micrograph may be due to the artifact generated when the vesicles were layering on the millipore filter under suction. Vesicles with double lamellae or more were occasionally seen, but they only account for a small percentage of the total population. The structure of lipid vesicles obtained from electron microscopy is consistent with the assumption that they are bound by a single bilayer of lipid.

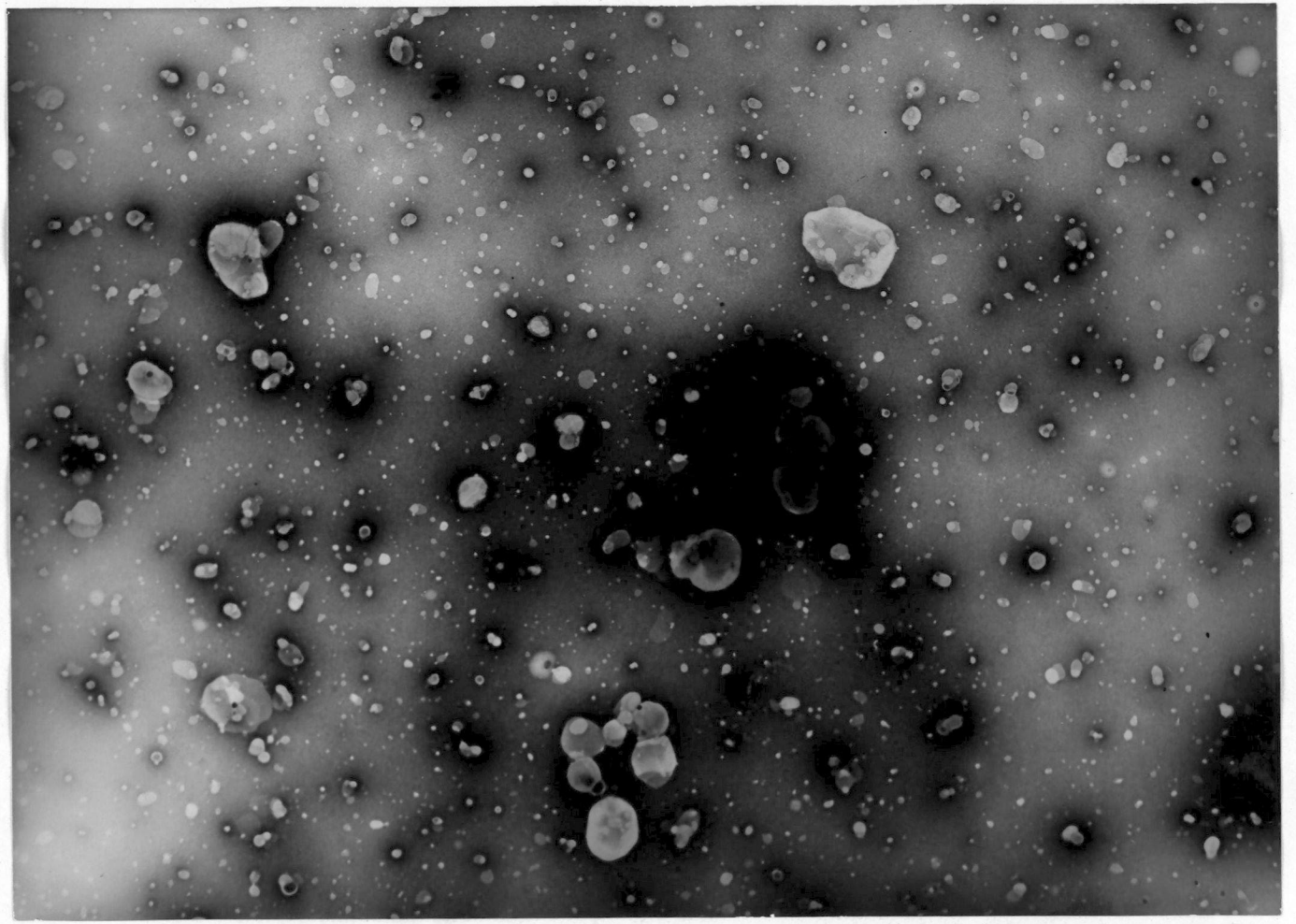


Figure 3. Negatively stained preparation of LUV before millipore filtration. (5,500X)

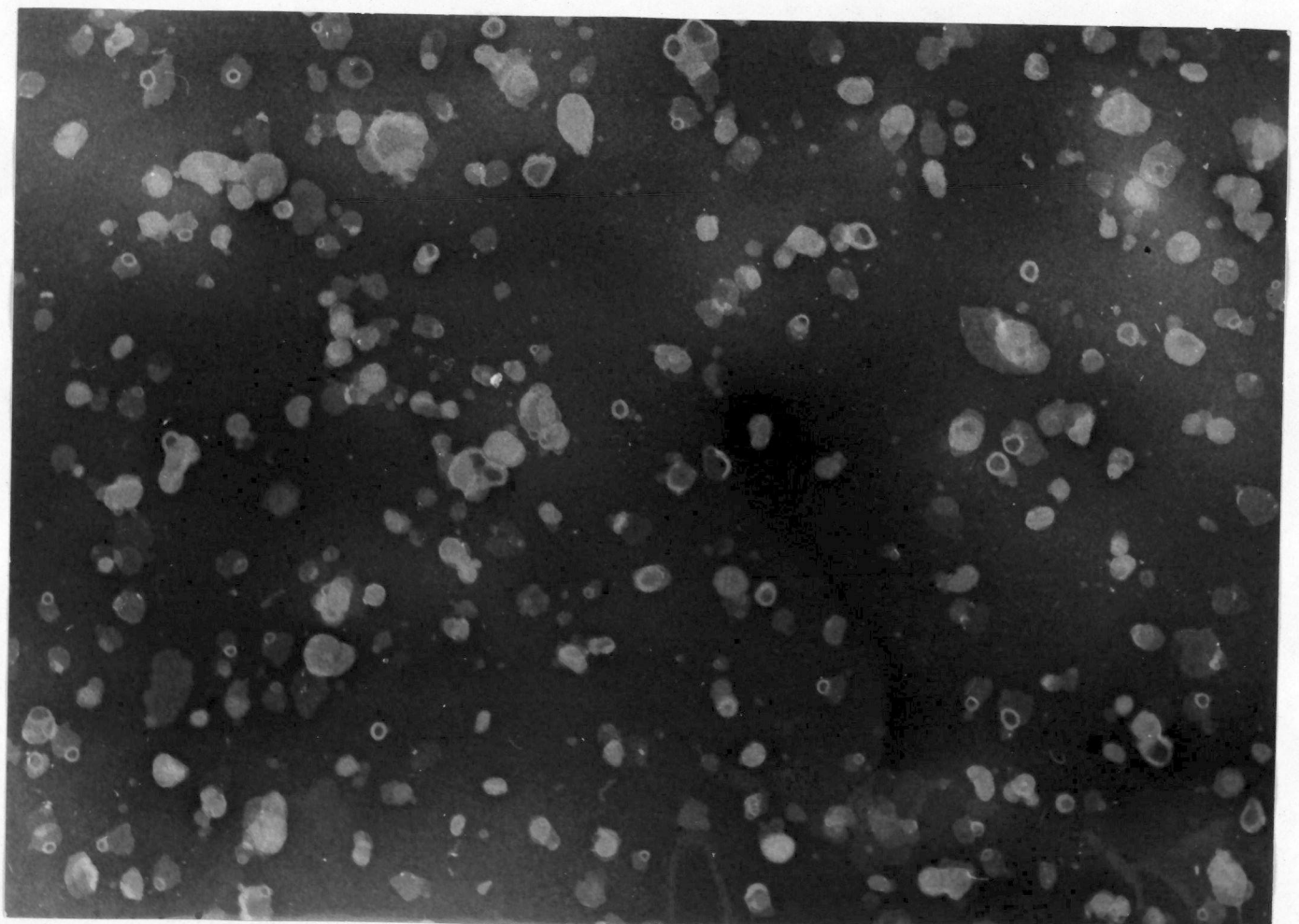


Figure 4. Negatively stained preparation of LUV after millipore filtration. (15,000X)

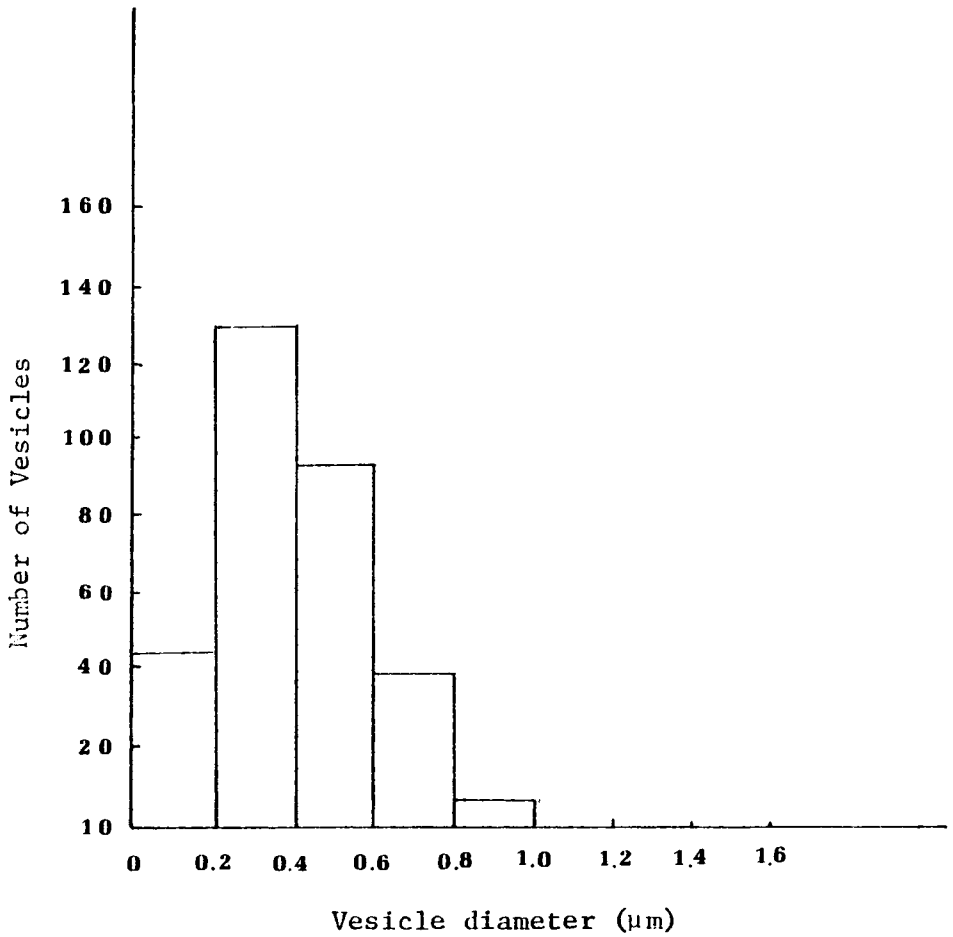


Figure 5. Size distribution of LUV, as determined by direct measurements from electron micrographs of the negatively stained vesicles.

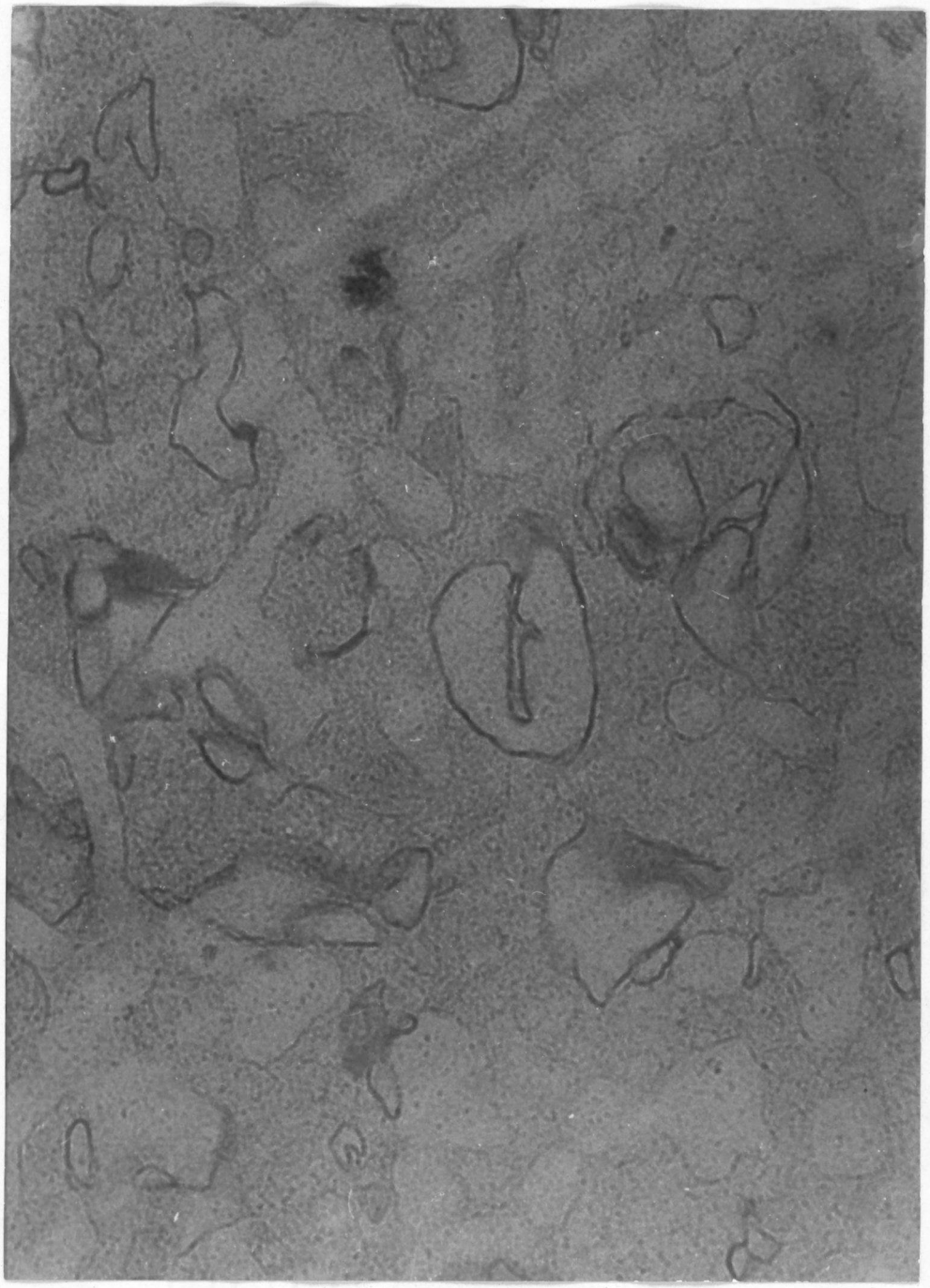


Figure 6. Thin-section electron micrograph of LUV. (55,000X)

In order to examine the potential of the LUV as carriers for therapeutic purposes, the trapping efficiency of LUV was compared with Deamer and Bangham's data (24) of SUV and MLV in Table II. The trapping efficiency of LUV is ten times higher than that of the SUV and five times higher than that of MLV.

TABLE II

THE TRAPPING EFFICIENCIES OF DIFFERENT PHOSPHOLIPID VESICLES

Sonicated SUV	0.8 ± 0.3 μl/μmol lipid
Hand-shaken preparation of MLV	1.8 ± 0.6 μl/μmol lipid
Lipid-ether injection preparation of LUV	10.2 ± 1.3 μl/μmol lipid

The suspension of unilamellar vesicles can be used in permeability studies especially for slowly permeating substances. Figure 7 shows the results of the permeability studies of LUV to the small nonelectrolytes, erythritol, malonamide and urea. The permeability coefficients (ω) and reflection coefficients (σ) were compared with that of Hill and Cohen's results of MLV in Table III. The LUV in this study were made up of 1:1 molar ratio of cholesterol to EYL. This was to slow the response by decreasing water per permeability of the vesicle membrane. The relative

Figure 7. The determination of nonelectrolyte permeability of LUV. The reciprocal of the rate of absorbance change was plotted as a function of the ratio of the outside concentration of impermeant (KCl) to permeant (nonelectrolyte) at 27°C. ⊖-⊖, erythritol; ✳-✳, malonamide; ●-●, urea.

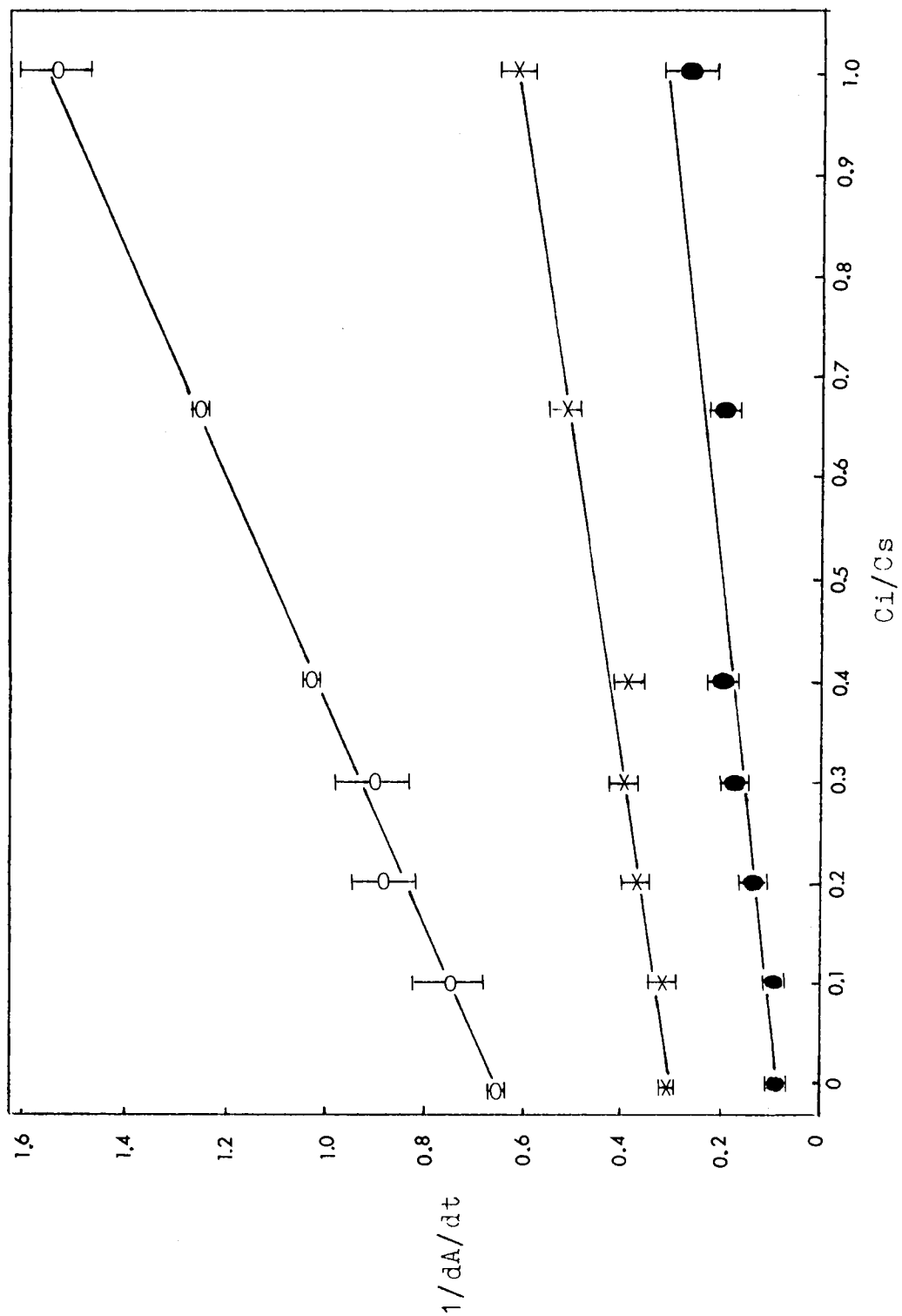


Figure 7

TABLE III

RELATIVE PERMEABILITY COEFFICIENT (ω) AND REFLECTION COEFFICIENT (σ) OF VESICLES TO NONELECTROLYTES

Nonelectrolyte	Present study 27°C		Hill and Cohen's Result+ 15°C		30°C	
	ω*	σ	ω	σ	ω	σ
Erythritol	1.0	0.71	1.0	0.66	1.0	0.38
Urea	7.2	0.40	9.5	0.23	7.6	0.14
Malonamide	2.1	0.98	4.0	0.34	3.2	0.23

+ Data are for MLV's, taken from (26).
* Expressed as relative values with respect to ω for erythritol.

permeability coefficients of these LUV were in the right order and were comparable to that of the MLV within experimental error. This results indicates that the permeability behavior of these LUV was similar to those of MLV. There were discrepancies when the reflection coefficients of the present result were compared with that of the Hill and Cohen. This may be due to the relatively long time required to mix the vesicle suspension with the hypertonic solution by hand which rendered the mesurements for the fast permeable solutes difficults. Stir within the cuvette has to be used in order to allow fast mixing. Nevertheless, the result of this experiment clearly demonstrated that LUV formed by ether injection were osmotically active.

CHAPTER IV

VESICLE-CELL INTERACTION

A. TEMPERATURE DEPENDENT UPTAKE OF LUV BY CHINESE HAMSTER V79 CELLS

Typical uptake curves for Chinese hamster V79 cells incubated at 37°C and 4°C at different time periods are shown in Figure 8. The kinetic of vesicle uptake appears to be similar to the uptake of SUV by the same cell type in other studies (20). V79 cells had a tremendous ability to incorporate lipid molecules without cytotoxic effects. Approximately 8×10^8 molecules of phospholipid per cell became cell-associated after 1 hr of incubation. The present study showed about 47% of the uptake is temperature independent. It is noted that EYL has a gel-liquid crystalline phase transition temperature (Tc) of -10°C. The two temperatures chosen for this study are well above the Tc for EYL. Therefore, the phospholipid bilayers of these vesicles were in a "fluid" state.

It is known that phagocytosis does not take place at a low temperature such as 4°C. Even at this temperature, considerable amounts of uptake occurred. This low-temperature insensitive uptake must be due to mechanisms other than phagocytosis.

B. ENERGY DEPENDENT UPTAKE OF LUV BY CHINESE HAMSTER V79 CELLS

Endocytotic activity, studied by Karnovsky (28) and Stainman et al. (29), has been demonstrated to be dependent on metabolic energy. Inhibitors can be used to cut off the energy supply and therefore block

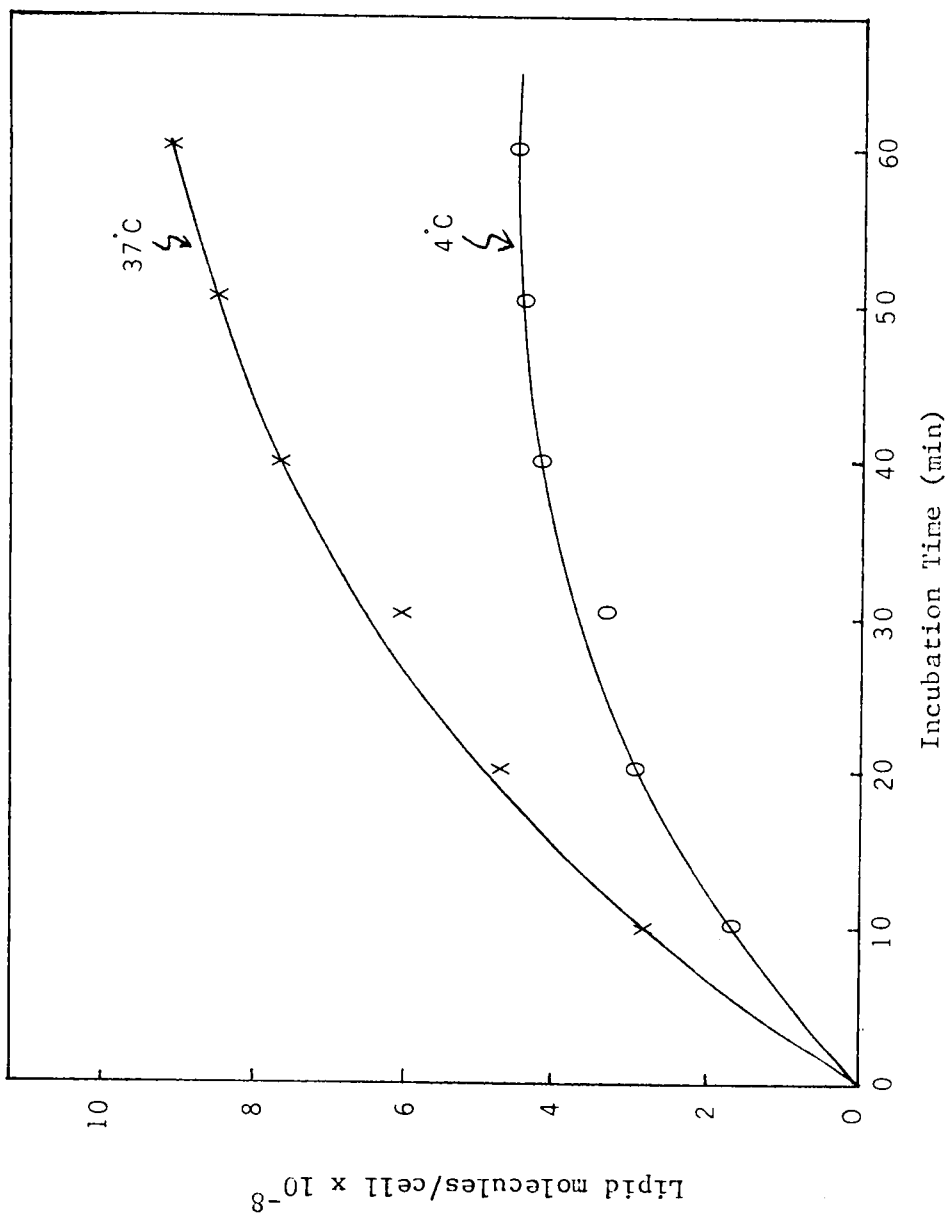


Figure 8. Uptake of ^{3}H-EYL by Chinese hamster V79 cells from LUV at 4°C (O—O), and 37°C (X—X).

endocytosis. The influence of various inhibitors on the uptake of phospholipid by V79 cells is summarized in Table IV. In the presence of a single metabolic inhibitor, either sodium azide (an inhibitor for respiration), 2,4-dinitrophenol (an inhibitor for oxidative phosphorylation), or 2-deoxyglucose (an inhibitor for glycolysis), about 30% or less of the uptake was inhibitable. If a combination of two inhibitors were used to block both respiration and glycolysis, 41% of the uptake was inhibitable. Therefore, it seems likely that a single metabolic inhibitor alone for either glycolysis or respiration cannot totally shut off all the energy-dependent uptake. In the presence of 0.1 mM cytochalasin B, a known inhibitor for endocytosis and cellular motility (30), a comparable amount of 30% of the uptake is inhibited. From these data it strongly suggested that a substantial fraction of the phospholipid uptake was not taken up by endocytosis and was independent of metabolic energy supply.

The effects of divalent cations and carbon source on the lipid uptake are shown in the last three columns of Table IV. Ca^{2+} and Mg^{2+} or D-glucose seems to have a slight stimulation on the uptake process, but when they were applied together in the vesicle suspension with the cells, 20% or more increase on the lipid uptake was achieved compared with the uptake without divalent cations and sugar. Sodium azide and 2-deoxyglucose could only inhibit about 17% of the uptake compared with control.

C. DIRECT DEMONSTRATION OF ENDOCYTOTIC ACTIVITY

If LUV are endocytosed by cells, the trapped content and the phospholipid of the vesicles are taken into the cell together. The

TABLE IV

EFFECT OF INHIBITORS, DIVALENT CATIONS, AND GLUCOSE ON PHOSPHOLIPID UPTAKE BY CHINESE HAMSTER V79CELLS TREATED WITH LUV*

	HCMF		HCMF + Ca^{2+}, Mg^{2+}		HCMF + glucose		HCMF + Ca^{2+}, Mg^{2+} + glucose	
	Uptake+	%	Uptake	%	Uptake	%	Uptake	%
Control	12.3	100.0	12.8	104.0	12.7	103.2	14.4	118.6
NaN_3 (30mM)	11.9	96.7	13.3	108.1	15.2	123.5	14.2	115.4
DNP (0.5mM)	8.4	68.2	13.3	108.1	10.3	83.7	16.1	130.8
Deoxyglucose (50mM)	8.5	69.1	11.4	92.6	10.2	86.1	14.6	118.6
Cytochalasin B (0.1mM)	8.6	70.6	9.2	74.7	8.9	72.3	12.7	103.2
NaN_3 + DNP	9.3	75.6	11.8	96.0	12.5	101.6	12.3	100.0
NaN_3 + Deoxyglucose	7.2	59.3	8.9	72.3	10.7	86.9	12.1	93.3

* Incubation was 1 hr at 37°C.

\+ Uptake is expressed as lipid molecules per cell X 10^{-8}.

localization of the lipid and the trapped content in the lysosomes will be a direct evidence for the endocytosis pathway.

Thin-section electron microscopy of cells incubated at 37°C with LUV revealed cytoplasmic vacuoles containing aggregated membranous material (Figure 9, arrows). This was not observed in cells without vesicles treatment or cells treated with vesicles in the presence of endocytosis inhibitor, cytochalasin B.

When cells were treated with BCIP-containing vesicles for 1 hr, blue granules were seen located within the cells under phase-contrast microscope (Figure 10), representing the entrapped marker was broken down, presumably by lysosomal acid phosphatase, and the end product formed was located inside the granules. The appearance of blue granules was not observed in cells treated with LUV without BCIP.

These two observations are compatible with the energy-dependent uptake of phospholipid. All these results directly demonstrated, although only qualitatively, that endocytosis of LUV was involved in the uptake of vesicles.

D. DEMONSTRATION OF FUSION

"Self-quenching" largely prevents the dye (6-CF) from fluorescing (89% of the fluorescence was quenched in 200 mM 6-CF) as long as it remains entrapped in vesicles. A diffused distribution of fluorescence throughout the cell may represent an enhancement of fluorescence when the dye is released from the vesicles and diluted into the much larger volume of the cell after vesicle-cell fusion.

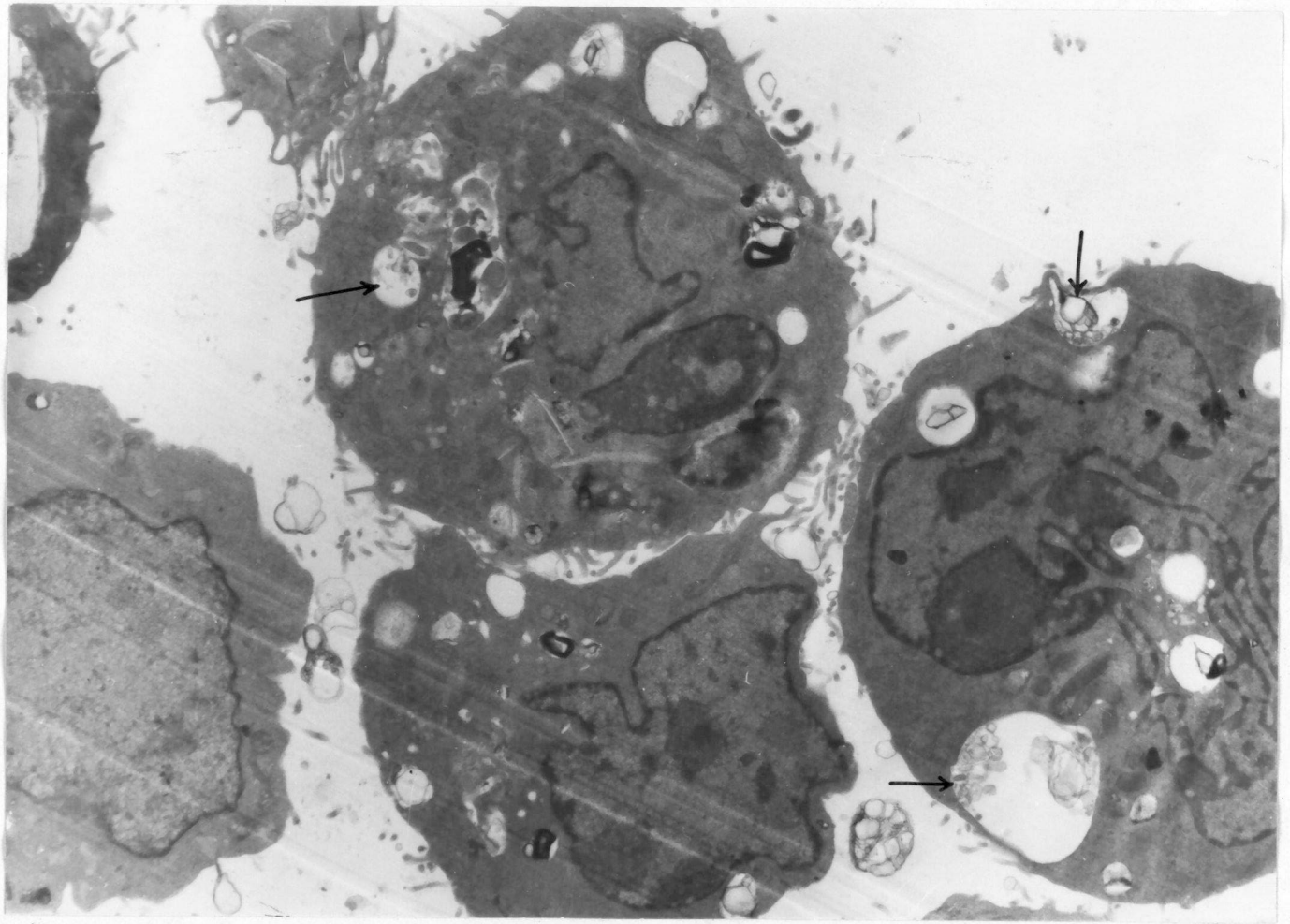


Figure 9. Electron micrograph of Chinese hamster V79 cells after incubation with LUV for 1 hr at 37°C. (530X) Arrows demonstrate the cytoplasmic vacuoles and secondary lysosomes containing membranous materials.

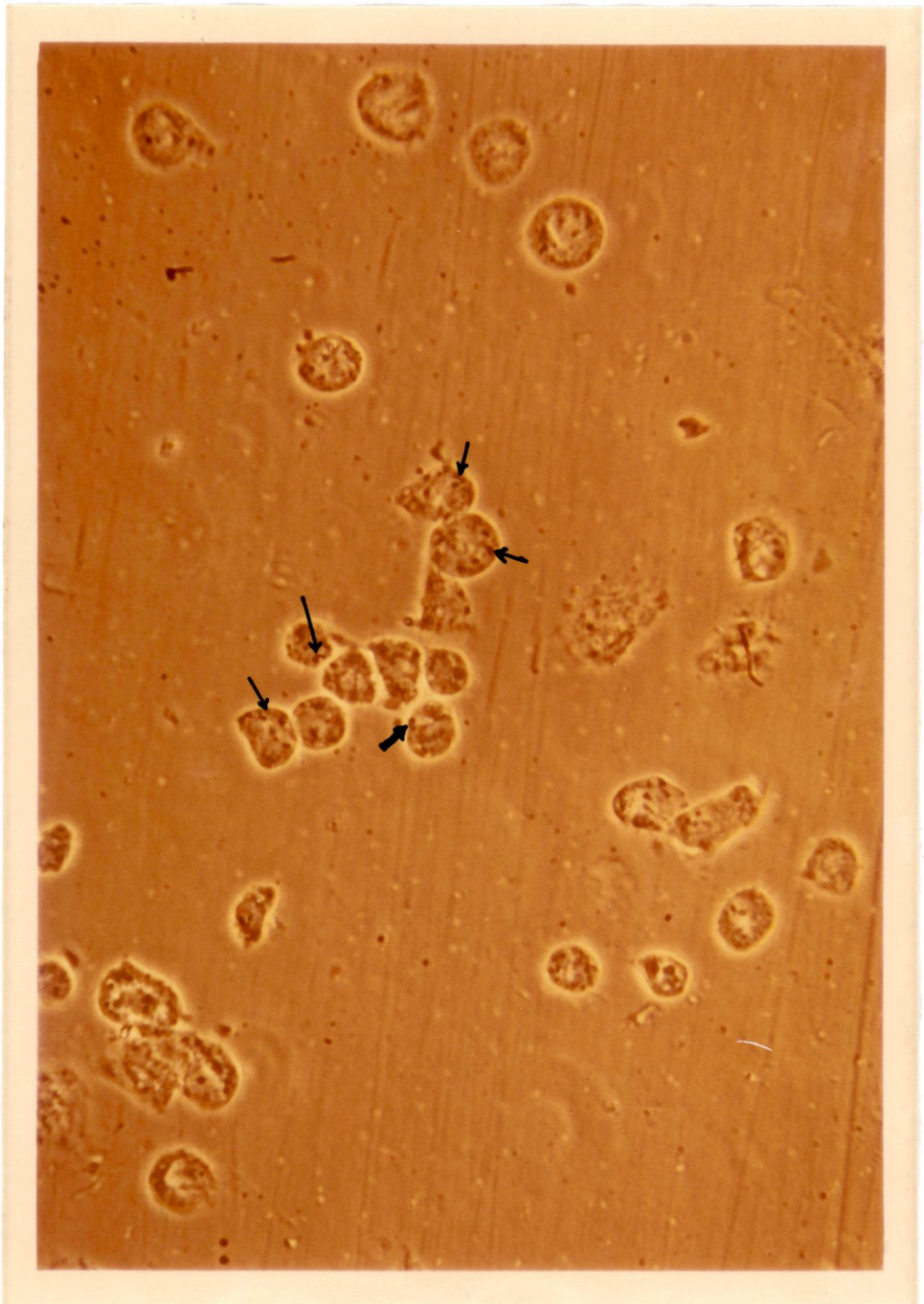


Figure 10. Photomicrograph of Chinese Hamster V79 Cells Treated with LUV containing BCIP.

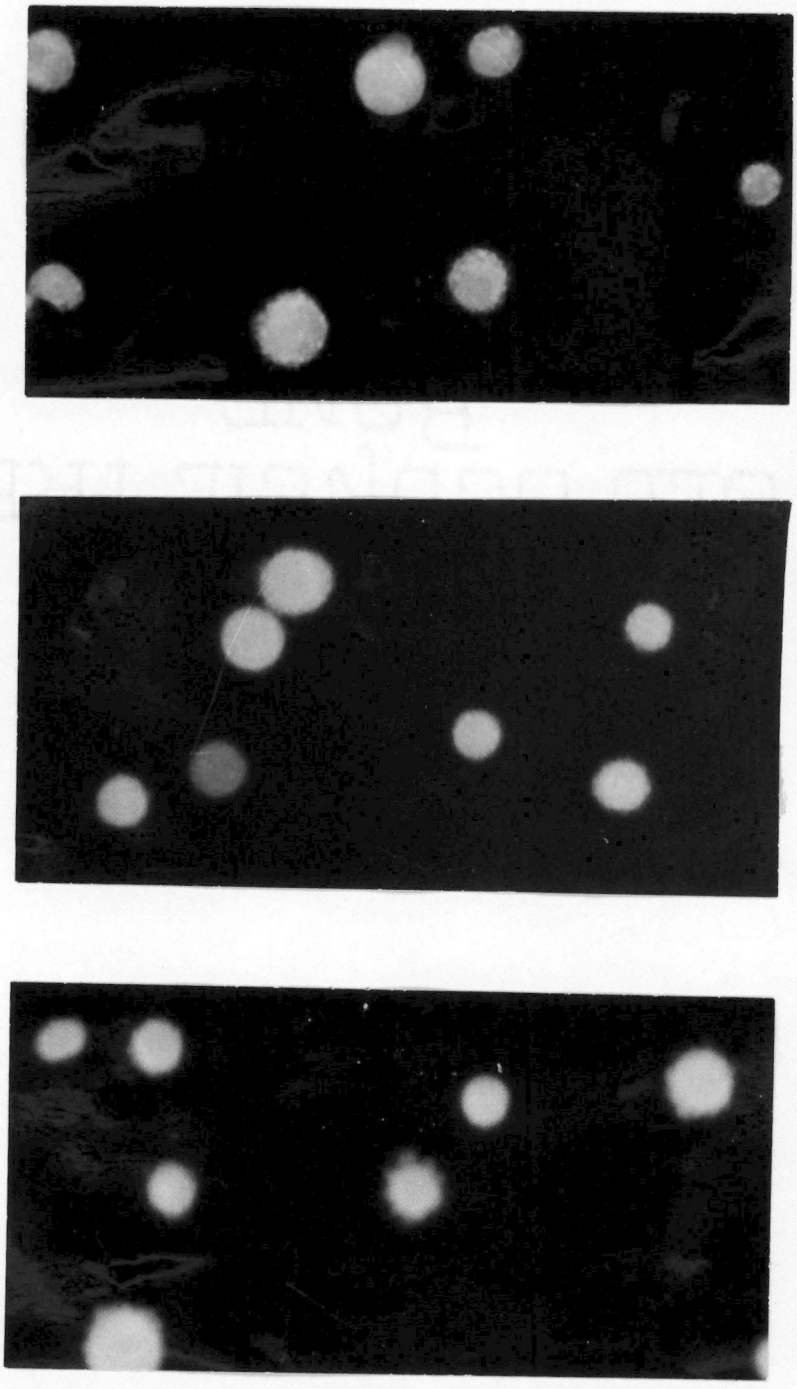
Cells treated with vesicles containing 200 mM 6-CF at 37°C for 2 hr showed a diffused, even distribution of fluorescence, with occasionally some bright fluorescent spots, inside the cells (Figure 11,a). Almost no fluorescence was seen in control populations of cells treated with empty vesicles together with free dye (200 mM).

Cells incubated with vesicles containing 6-CF in the presence of cytochalasin B (Figure 11,b) or a combination of sodium azide and deoxyglucose (Figure 11,c) showed a similar diffused, even distribution of dye fluorescence, only without the bright fluorescent spots within the cells.

The finding of a uniform fluorescence in cells indicates the release of this dye into the cytoplasmic space. This observation is consistent with the fusion mechanism. Localized accumulation of fluorescence dye present in cells (bright spots) without inhibitor treatment may have represented secondary lysosomes.

Furthermore, when cells treated with vesicles containing 20 mM 6-CF, very little of fluorescence was seen with the cells (data not shown). At this concentration 6-CF, the vesicles are fluorescent, and if vesicles are attached at the cell surface, fluorescence at cell periphery should be observed. This suggested that absorption of intact LUV on the cell surface does not involve the vesicle uptake.

These results showed that fusion could also be responsible for some of the vesicle uptake.



(a)

(b)

(c)

Figure 11. Fluorescence micrographs of Chinese hamster V79 cells incubated with 6-CF-containing LUV for 2 hr at 37°C. (a); in the presence of 30 mM sodium azide and 50 mM deoxyglucose (b); in the presence of 0.1 mM cytochalasin B (c).

E. UPTAKE OF TRAPPED CONTENT AND VESICLE MEMBRANE BY V79 CELLS

In order to examine whether the lipid uptake represents the uptake of intact vesicles or not, cells were treated with double-labeled LUV using ^{3}H-methoxy-inulin as trapped content marker and ^{14}C-EYL for the lipid component. Uptakes of these two vesicle components are shown in Figure 12. The dashed line represents the theoretical amount of ^{3}H-methoxy-inulin that should have become cell associated if both the vesicle lipid and trapped marker enter the cell together. This line was calculated from the observed uptake of ^{14}C-EYL and the ratio of ^{3}H cpm/^{14}C cpm in the applied vesicle suspension. The data showed that the rates of uptake for these two vesicle components were different. After 1 hr of incubation, the amount of trapped marker taken up only represents 13% of the theoretical amount of uptake. The result of this experiment suggested that the majority of the lipid uptake was not a direct result of intact vesicles uptake. The mechanisms, such as endocytosis, adsorption or non-leaky fusion require that both of the lipid component and the trapped aqueous component of the vesicles should become cell-associated at the same rate. The present data showed that a large portion of the lipid uptake was independent of the trapped-content uptake. Therefore, mechanisms such as endocytosis, adsorption or non-leaky fusion are eliminated as the major pathway for the lipid uptake, although these mechanisms may still be responsible for a minor amount of lipid uptake.

F. UPTAKE OF LIPID COMPONENT OF LUV BY V79 CELLS

The same kind of strategy was used to examine whether the lipid

Figure 12. Uptake of ^{14}C-EYL and ^{3}H-methoxy-inulin by V79 cells treated with ^{3}H-methoxy-inulin-entrapped LUV at 37°C. X—X, ^{14}C-EYL, 0—0, ^{3}H-methoxy-inulin; ----, theoretical uptake of ^{3}H-methoxy-inulin if its uptake rate is the same as that of ^{14}C-EYL.

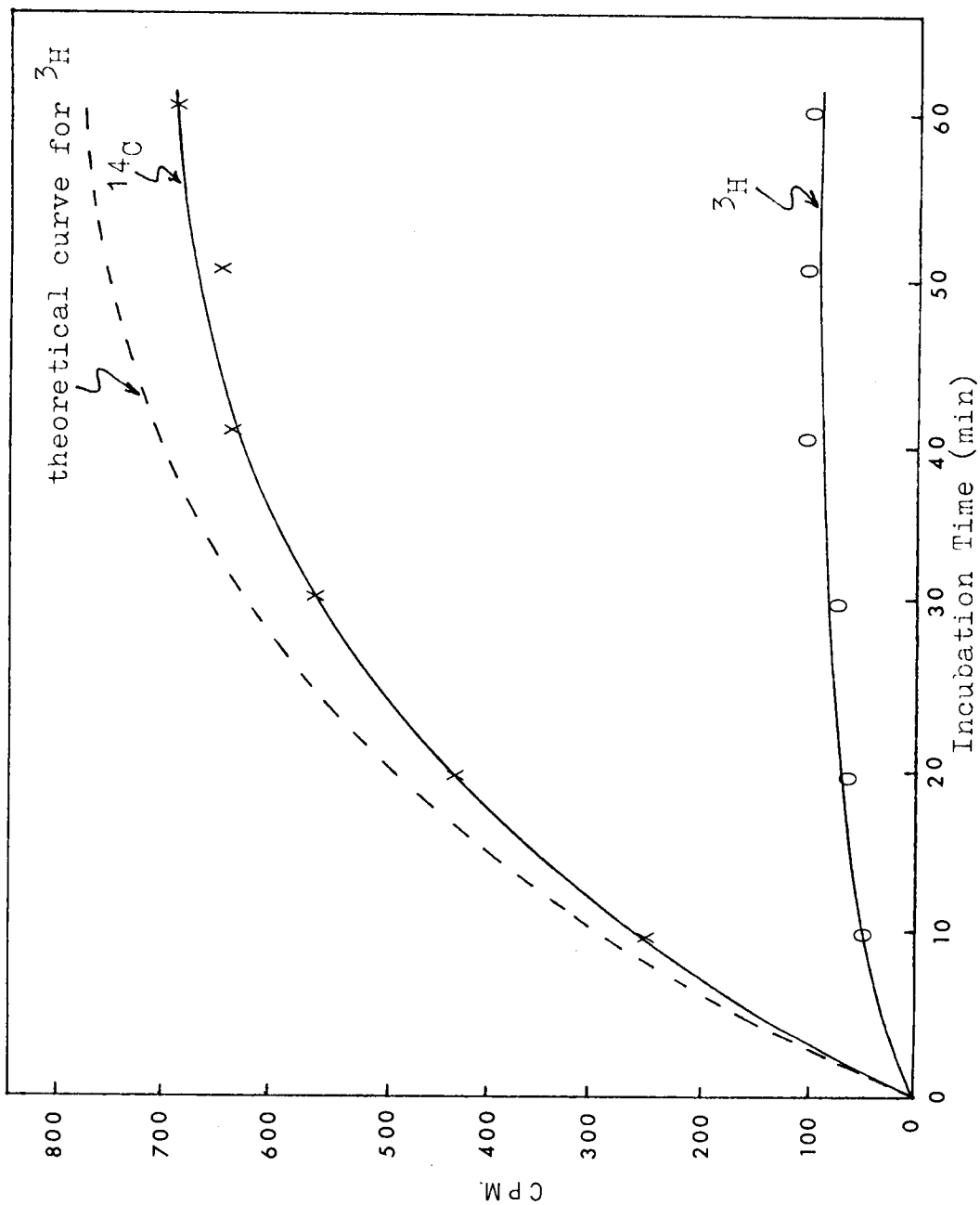

theoretical curve for ^{3}H
^{14}C
^{3}H
CPM.
Incubation Time (min)
0
100
200
300
400
500
600
700
800
10
20
30
40
50
60

Figure 12

components of vesicles were taken up as a whole. The vesicles were labeled with ^{3}H-EYL and trace amount of ^{14}C-cholesterol. The uptake of the phospholipid and cholesterol is shown in Figure 13. Again, the dashed line represents the theoretic uptake of cholesterol if its uptake was at the same rate as phospholipid uptake. The uptake of cholesterol only represents 16% of the theoretical value. This clearly demonstrated that only a small portion of the vesicle lipid was taken up as intact structure. This experiment also eliminates leaky fusion as the major pathway for vesicle uptake, in which components of vesicle membranes are expected to become cell-associated at the same rate.

G. MECHANISMS OF VESICLE-CELL INTERACTION

From all the experimental data mentioned above, it is known that the interaction of LUV composed of EYL with V79 cells is not a simple process. Possible mechanisms of vesicle-cell interaction are shown in Figure 14.

Five possible mechanisms of vesicle-cell interaction are discussed. (1) In stable adsorption, the vesicles are attached to the cell periphery and they remain as intact vesicles. (2) Endocytosis is when the whole vesicle is engulfed by the cell with the formation of the cytoplasmic vacuoles. These vacuoles are subjected to being attacked by the lysosomes. (3) In non-leaky fusion, vesicles fuse with the plasma membrane of the cell with the release of all the entrapped materials into the cytoplasmic space. The entrapped materials are expected to be evenly distributed throughout the cytoplasm. All the three mechanisms mentioned above require that the vesicle membrane and the trapped content become cell-associated at the same rate. (4) In leaky fusion, the vesicle

Figure 13. Uptake of ^{3}H-EYL and ^{14}C-cholesterol by V79 cells from LUV at 37°C. Trace amount of ^{14}C-cholesterol was present in the membranes of LUV. X—X, ^{3}H-EYL; 0—0, ^{14}C-cholesterol; ----, theoretical uptake of ^{14}C-cholesterol at the same rate of ^{3}H-EYL.

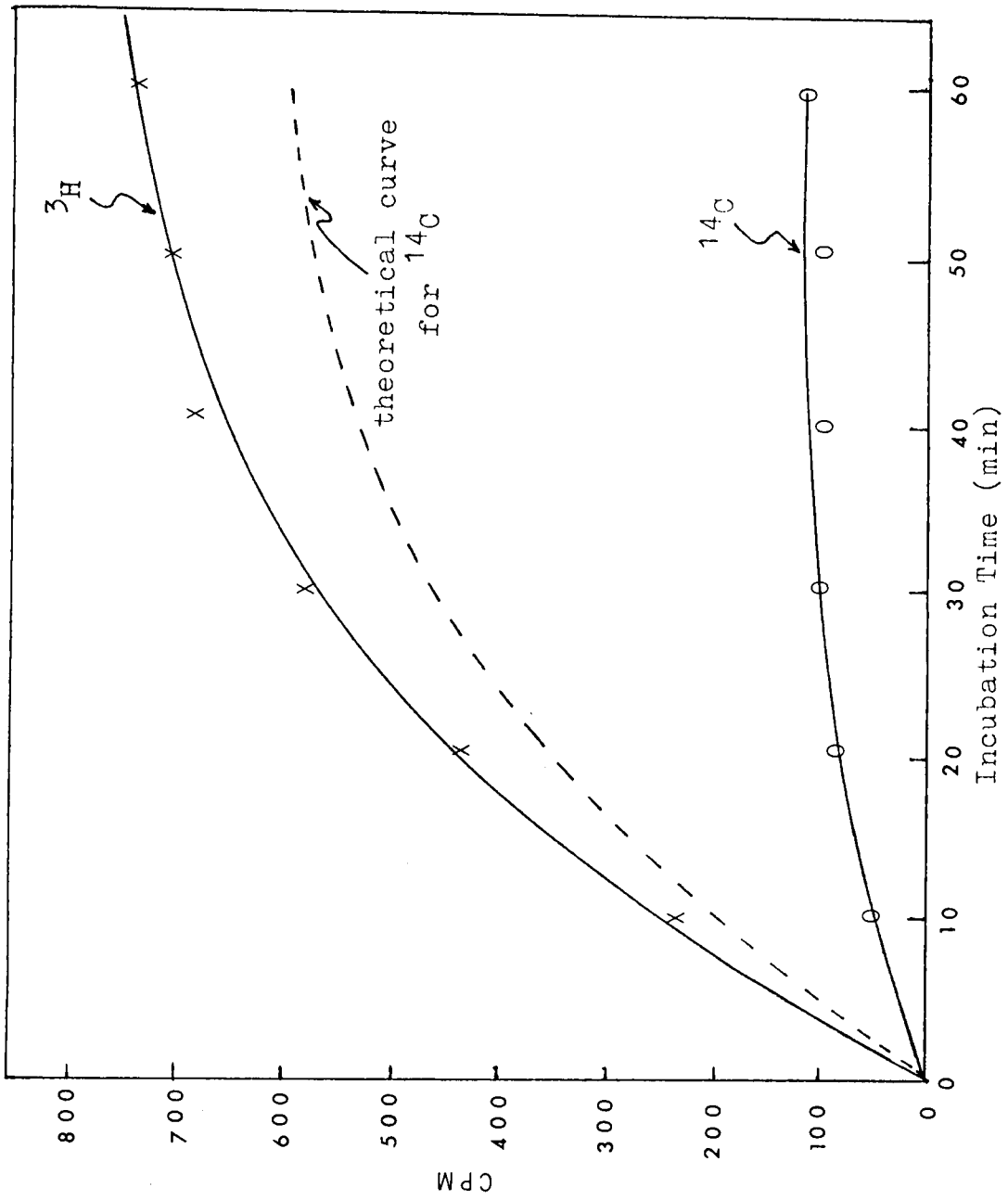

^3H
theoretical curve for ^{14}C
^{14}C
CPM
Incubation Time (min)
0
100
200
300
400
500
600
700
800
10
20
30
40
50
60

Figure 13

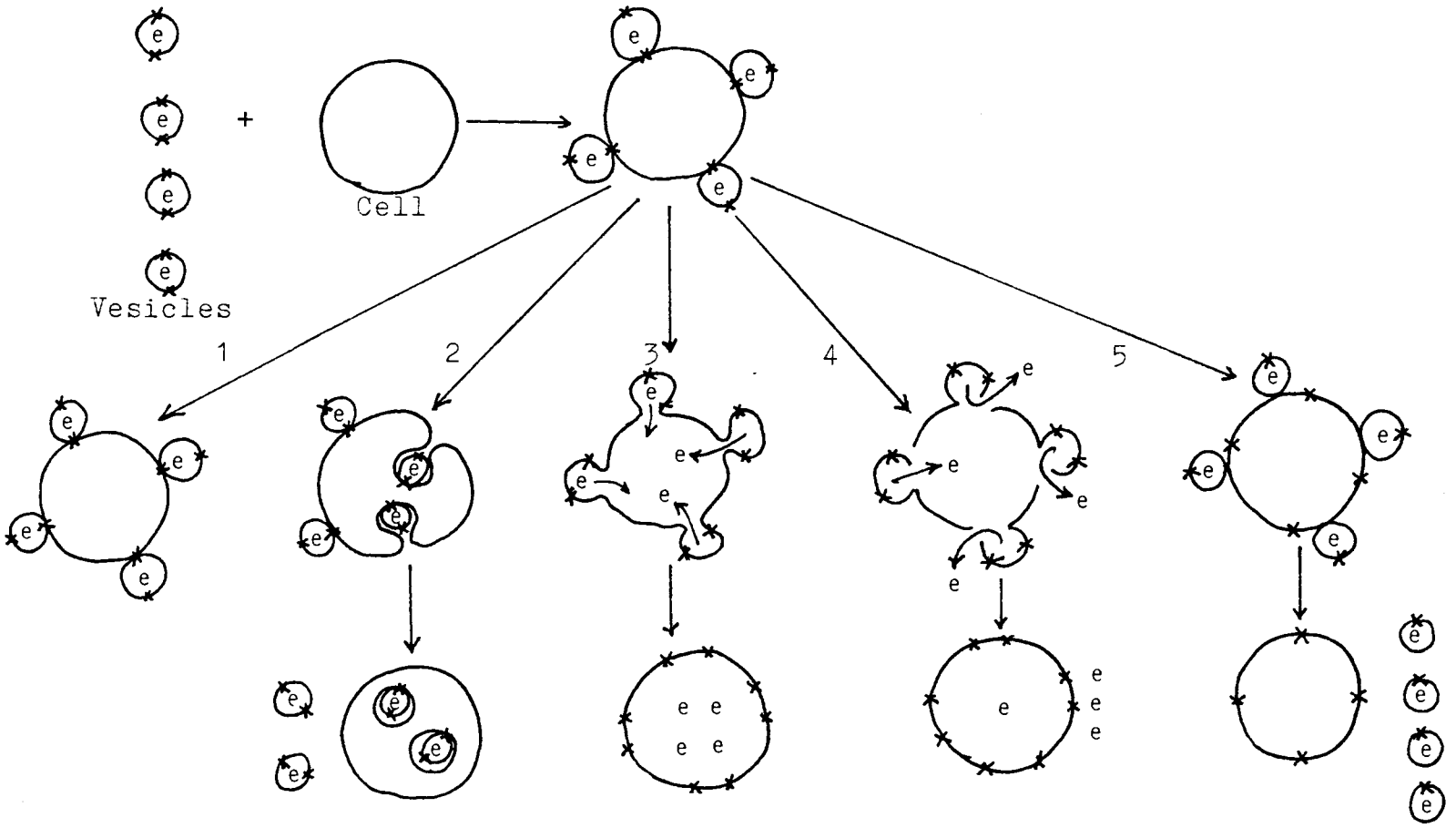


Figure 14. Different possible mechanisms for vesicle-cell interaction. x, lipid marker; e, trapped content marker; 1, stable adsorption; 2, endocytosis; 3, non-leaky fusion; 4, leaky fusion; 5, lipid transfer.

membrane fuses with the plasma membrane of the cell and becomes part of the plasma membrane. Dring the rearrangement of the lipid molecules of the two membranes, the trapped content in the vesicles leaks out into the medium. Therefore, the rate of vesicle membrane uptake is different from that of the trapped material uptake. (5) In lipid transfer, the transfer of lipid molecules from the vesicle to the cell without disrupting the vesicle. As a result, only the lipid molecules are taken up by the cell.

V79 cells can take up a large amount of phospholipid without impairing the viability. A minor amount of the lipid uptake was sensitive to low temperature and metabolic energy inhibitors. Further study with electron microscopy showed aggregated membranous material in the cytoplasmic vacuoles or lysosomes, which can be interpreted as the result of endocytotic activity by localizing the sites of enzymatic digestion of the vesicle-entrapped marker in the organelles.

Fusion was also involved in vesicle uptake. A uniform distribution of vesicle-entrapped fluorescent dye, 6-CF, within the cell is interpreted as the release of the trapped content into the cytoplasm after vesicle-cell fusion. Adsorption did not contribute to the vesicle uptake due to the fact that vesicles containing low concentration of fluorescent dye were not seen on the cell curface. This conclusion is also suuported by the electron microscopic observations of vesicle-treated cells.

The phospholipid taken up by endocytosis and non-leaky fusion only contributes a small proportion of the total vesicle uptake. Eighty-seven percent of the phospholipid uptake was independent of the uptake of the trapped content, suggesting that this uptake could not be the result of

endocytosis, adsorption or non-leaky fusion. Further experiment showed that the vesicle membrane was not taken up as a whole. Among the five different mechanisms proposed, phospholipid transfer is the only mechanism that fits all the experimental results obtained. Although direct evidence to support lipid transfer is not available, it is tentatively concluded that this mechanism is responsible for the major portion of lipid uptake observed in the present studies of LUV-cell interactions.

CHAPTER V

SUMMARY

Lipid vesicles offer a new and poverful technique for achieving cellular uptake of materials that would not ordinarily be taken up by cells. In the present study, a method of preparing large unilamellar vesicles is described. These vesicles are homogeneous in size with an average diameter of 0.2 to 0.4 μm. They have a high trapping efficiency and are osmotically active.

The interactions of Chinese hamster V79 cells with EYL vesicles were studied in vitro. The different possibilities of interaction complicate the system. The rates for the uptake of the vesicle content and the lipid membrane are different, arguing against endocytosis, non-leaky fusion and adsorption to be the major pathway for the vesicle lipid uptake. The differential uptake of the membrane components of the vesicle by the cells further argues against leaky fusion to be the major process. Among the five different mechanisms proposed, lipid transfer is therefore the most possible mechanism responsible for the majority of vesicle lipid uptake.

It has been suggested that lipid vesicles can interact with the cell surface through lipid-lipid interaction or lipid-protein interaction (32). The recent discovery of the phospholipid exchange protein may suggest that phospholipid exchange can be a protein-mediated process. Direct evidence to rigorously demonstrate phospholipid exchange to be the major pathway for the lipid uptake will require further experiments.

It is known that SUV, made of EYL interact with V79 cells mainly through fusion (20). In this study, LUV of the same lipid composition seem to interact with the same cell type by lipid transfer. This difference may suggest that the degree of curvature and packing of lipid molecules in the membrane bilayers at the point of vesicle-cell contact may be important factors to govern different pathways or interactions.

Endocytosis of LUV by the cells was also demonstrated. This is based on the observations that a portion of the phospholipid uptake was temperature-sensitive and energy dependent. Electron microscopic and histochemical studies showed direct evidence for this pathway.

Fluorescence microscopy of cells treated with vesicles containing a water-soluble dye, 6-CF, showed a diffused, even distribution of fluorescence throughout the cells, providing evidence for vesicle-cell fusion.

It is therefore concluded that LUV can be employed as a carrier to deliver biologically active substances into various subcellular compartments, and thus are potentially useful as a therapeutic vector.

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VITA

Siu-Cheong Ho was born in Hong Kong on September 25, 1948. He attended elementary schools in that city and was graduated from Literary College in May 1971. The following June, he came to the United States to pursue higher education in Austin Peay State University in Clarksville, Tennessee. In March 1974, he received a Bachelor of Science degree with a major in Chemistry. In the same month, he enrolled in the graduate program offered by the Biochemistry Department in the University of Tennessee in Knoxville, Tennessee. He received a Master of Science degree in that department in December 1977.

He was married to his high school sweet-heart, Kwai-Pheng Wong in September 18, 1974.