To the Graduate Council:

I am submitting herewith a thesis written by Emily Tate Brake entitled "Pinocytosis in HeLa Suspension Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

James N. Dumont

James N. Dumont, Major Professor

We have read this thesis and recommend its acceptance:

Frank Gaither

John S. Cook

Accepted for the Council:

L. Evans Roth

Vice Chancellor
Graduate Studies and Research

Thesis
77
.B729
cop. 2

PINOCYTOSIS IN HELA SUSPENSION CELLS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Emily Tate Brake

December 1977

1345645

ACKNOWLEDGMENTS

The author wishes to thank the members of her committee, James N. Dumont, Frank H. Gaertner, and John S. Cook, for their assistance. Special thanks go to John Cook for his guidance, encouragement, and help with this manuscript. The author thanks Don Salter for his helpful suggestions related to some of the experiments, to Peggy Braden for her dedicated assistance with some of the lengthy experiments, to Mitchel Villereal for his helpful comments about the manuscript, to Shirley Sands and Neva Hair for their excellent typing assistance, and to Carole Richter for her help with the SEM prints.

Lastly, but most importantly, the author expresses her deep appreciation to her husband, Rick, for his understanding, patience, instruction, and helpful suggestions about this manuscript.

ABSTRACT

L-glucose, D-mannitol, horseradish peroxidase (HRP), sucrose, and inulin are evaluated as possible markers for nonadsorptive pinocytosis in HeLa S3 suspension cells. Of these compounds, sucrose and inulin are the markers which best meet the criteria given. The influx rates, intracellular fates, and efflux rates are determined for these two molecules. The uptake of sucrose and inulin corresponds to 2.5% of the cell volume (~0.05 pl) per hr, and is linear for about 10 hrs. This is approximately 10% of the primarily diffusional uptake of L-glucose or D-mannitol. Influx of sucrose and inulin is temperature sensitive, and uptake measured at 4°C is 10%-15% of that measured at 37°C. Pinocytic uptake of sucrose and inulin was further classified as micropinocytosis (uptake into vesicles 0.07-0.1 μm in diameter), since the uptakes are insensitive to ATP depletion and microfilament disruption.

Ouabain, which binds to the Na^+, K^+ ATPase in the plasma membrane, was chosen as a marker for adsorptive pinocytosis. Its influx rate shows that one complete cell surface is endocytosed every 15 hr. Cell fractionation experiments show that > 90% of internalized ouabain is associated with sedimentable cellular material, and that it codistributes with the lysosomal marker enzyme, N-acetyl-β-glucosaminidase. Similar fractionation experiments show that 20% and 50% respectively of the total intracellular sucrose and inulin is sedimentable. Intracellular sucrose, inulin, and ouabain are not metabolized by HeLa cells. The efflux of sucrose and inulin is best described by two-component kinetics. A rapid

efflux component represents about 18% of the total sucrose efflux and approximately 6% of the total inulin efflux. Average half-times for the rapid efflux component are 40 and 60 min for sucrose and inulin respectively. The slow efflux component for sucrose has a half-time of 54 hr and for inulin, 125 hr. Ouabain efflux is first order, and its half-time of 60 hr is similar to that of the slow component of sucrose efflux. Neither micropinocytic rate, determined by sucrose and inulin influx measurements, nor cell surface morphology, determined by scanning electron microscopy (SEM), changes during the cell cycle.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	5
III.	KINETICS OF PINOCYTIC MARKER UPTAKE	16
IV.	INTRACELLULAR LOCALIZATION AND METABOLISM OF PINOCYTIC MARKERS	37
V.	EFFLUX OF PINOCYTIC MARKERS	48
VI.	MICROPINOCYTOSIS DURING THE CELL CYCLE	60
VII.	PREPARATION OF SUSPENSION-CULTURED MAMMALIAN CELLS FOR SCANNING ELECTRON MICROSCOPY (SEM)	64
VIII.	SUMMARY AND OVERVIEW	82
BIBLIOGRAPHY		87
VITA		98

LIST OF TABLES

TABLE		PAGE
I-1.	Definitions of Commonly Used Terms for Uptake by Vesicle Formation	2
III-1.	Uptake Rates of Several Commonly Used Pinocytic Markers	17
III-2.	Effect of Several Experimental Treatments on the Rapid Sucrose Influx	21
III-3.	Effect of Polyamino Acids on Several Parameters of Viable Cells	26
III-4.	Uptake Rates of Putative Micropinocytic Markers	28
III-5.	Effect of ATP Depletion, Cytochalasin B, and Cold on Micropinocytosis of Sucrose and Inulin	35
IV-1.	Cellular Distribution of Pinocytic Markers	42
V-1.	Average Half-times for Pinocytic Marker Efflux	49
VII-1.	A Comparison of Volumes Measured with the Coulter Channelyzer and Volumes Calculated from Diameters Measured with the SEM	71

LIST OF FIGURES

FIGURE		PAGE
III-1.	Comparison of Sucrose and L-Glucose Uptake in HeLa Suspension Cells	19
III-2.	Dependence of the Rapid Sucrose Influx on Cell Viability	23
III-3.	A Comparison of D-Mannitol and Sucrose Uptake in the Presence and Absence of ATP Depleting Agents	30
III-4.	Long Term Uptake of Ouabain in HeLa Suspension Cells	32
IV-1.	Dependence of Ouabain Pelletability on Number of Homogenization Strokes	39
IV-2.	Codistribution of Internalized Ouabain and N-acetyl-β-glucosaminidase Activity	41
IV-3.	Chromatography of Cell Extracts from [^{14}C]Sucrose-loaded Cells	45
IV-4.	Chromatography of a Lysate of Inulin-loaded HeLa Cells	46
V-1.	Sucrose Efflux from HeLa Suspension Cells after Different Influx Times	50
V-2.	Efflux of Inulin from HeLa Suspension Cells	52
V-3.	A Comparison of Sucrose and Internalized Ouabain Release from HeLa Suspension Cells	53
V-4.	Centrifugation and Resuspension of [^{14}C]Sucrose-loaded HeLa Cells Does Not Induce Component F of Sucrose Efflux	55
V-5.	Micropinocytosis of Sucrose in HeLa Cells and a Model for Component F of Sucrose Efflux	56
VI-1.	HeLa Suspension Cell Volume, Number, and Micropinocytic Rate during the Cell Cycle	61
VI-2.	Micropinocytosis of Inulin and Sucrose during the Cell Cycle	63

FIGURE PAGE

VII-1. Scanning Electron Micrograph of Control HeLa Suspension Cells 69

VII-2. Scanning Electron Micrograph of Experimentally Swelled HeLa Suspension Cells 70

VII-3. Osmotic Responsiveness of HeLa Suspension Cells after Glutaraldehyde Fixation 73

VII-4. The Effect of Ethanol Dehydration on HeLa Cell Volume . . . 75

VII-5. Scanning Electron Micrographs of HeLa Suspension Cells at Different Stages of the Cell Cycle 78

CHAPTER I

INTRODUCTION

Uptake of extracellular materials within membrane-limited vesicles is called endocytosis (De Duve, 1963). The vesicles arise from invaginations of the plasma membrane, which then break free of the membrane on its cytoplasmic side and move into the cell. There are two major types of endocytosis (Table I-1). The first type is phagocytosis which refers to the uptake of solid particles, e.g. bacteria and latex beads, into vesicles $\geq$ 1 μm in diameter following adsorption of the particles to the cell surface (Allison and Davies, 1974). The uptake step of phagocytosis but not the adsorption step is inhibited by cellular ATP depletion, by microfilament disruption with cytochalasin B, or by low temperature (Casley-Smith, 1969; Davies et al., 1973c). Although there must necessarily be some fluid uptake during phagocytosis, transmission electron micrographs show that the amount of space occupied by fluid in phagocytic vesicles is small compared to that taken up by the solid particle (Casley-Smith, 1969; Davies et al., 1973c).

The second type of endocytosis, called pinocytosis, was first described by Lewis (1931) and refers to uptake of extracellular fluid and its solutes by membrane invagination and vesiculation as described above. Allison and Davies (1974) have subdivided pinocytosis into categories based on vesicle size and dependence on cellular ATP levels and intact microfilaments (Table I-1). By their definition, macropinocytosis is fluid uptake into relatively large vesicles (usually

TABLE I-1

DEFINITIONS OF COMMONLY USED TERMS FOR UPTAKE BY VESICLE FORMATION

Term	Definition	Vesicle Size Range
Endocytosis	Uptake by membrane-limited vesicles	0.07-2 μm
Phagocytosis	Uptake of solid particles by adsorption followed by endocytosis	$\geq$ 1 μm
Pinocytosis	Uptake of fluid and its solutes by endocytosis	0.07-2 μm
Macropinocytosis	Uptake of fluid and its solutes by endocytosis	0.3-2 μm
Micropinocytosis	Uptake of fluid and its solutes by endocytosis	0.07-0.1 μm
Adsorptive Pinocytosis	Uptake of an adsorbed molecule by pinocytosis	
Adsorptive Macropinocytosis	Uptake of an adsorbed molecule by pinocytosis	0.3-2 μm
Adsorptive Micropinocytosis	Uptake of an adsorbed molecule by pinocytosis	0.07-0.1 μm

0.3-2 μm in diameter) which are visible by light microscopy. This process is inhibited by ATP depletion or microfilament disruption (Cohn, 1970; Wills et al., 1972; Casley-Smith, 1969). Micropinocytosis is fluid uptake in vesicles with a diameter of 0.07-0.10 μm (Allison and Davies, 1974). These investigators found that the lower vesicle size limit (0.07 μm) was relatively constant indicating that this size may be the minimum diameter of a sphere that can readily be formed by distortion of a biological membrane. Micropinocytosis is not energy- or microfilament-dependent (Casley-Smith, 1969; Wills et al., 1972). In some systems, it is not even inhibited by low temperatures (Casley-Smith, 1969).

When binding of a molecule to the plasma membrane precedes its uptake by pinocytosis, the ingestion process is called adsorptive pinocytosis (Jacques, 1969). This process can be further subdivided by vesicle size and energy and microfilament dependence into adsorptive micropinocytosis and macropinocytosis (Table I-1).

The study of these endocytic processes requires specific markers. These are commonly radioactive tracers (Wagner et al., 1971; Ryser, 1967a; Ehrenreich and Cohn, 1968; and Williams et al., 1975a, 1975b) or substances which are electron dense (Petitpierre-Gabathuler et al., 1975) or give electron dense reaction products (Steinman and Cohn, 1972; Steinman et al., 1974). Radioactive tracers are convenient for measuring endocytic rates, while electron dense tracers are more useful for localization and other ultrastructural studies using the transmission electron microscope.

The purpose of the present study was to evaluate several possible markers for nonadsorptive pinocytosis in HeLa S3 suspension cells. The influx rates (in exponentially growing and synchronized cells), the intracellular fates, and the efflux rates were determined for those molecules that were evaluated as good markers. The latter parameters were also measured using a marker for adsorptive pinocytosis. Radioactive tracers were used because the experiments involved primarily rate measurements. The criteria employed in evaluating the markers of nonadsorptive pinocytosis were as follows: (1) the marker should be nontoxic, stable, and its uptake easily assayed; (2) there should be no plasma membrane transport system which utilizes it as a substrate; (3) the plasma membrane should be relatively impermeable to the marker so that the diffusion component of uptake is very small; (4) the marker should not bind to the cells; (5) the marker should not stimulate or inhibit pinocytosis; and (6) the marker should not be metabolized by the cell, since this would complicate influx and efflux rate measurements.

Sucrose, inulin, horseradish peroxidase (HRP), L-glucose, and D-mannitol were chosen as possible markers for nonadsorptive pinocytosis, and experiments demonstrated that inulin and sucrose best meet the criteria described above. Ingestion of these molecules was classified as micropinocytosis because their uptakes were insensitive to microfilament disruption and ATP depletion. In contrast, uptake of ouabain, a specific inhibitor of the membrane Na^+, K^+ transport system, was classified as adsorptive macropinocytosis, since its uptake involves prior binding to the Na^+, K^+ ATPase and is sensitive to ATP depletion.

CHAPTER II

MATERIALS AND METHODS

Materials

Eagle's minimal essential medium with Spinner salts (S-EMEM) and heat-inactivated fetal calf serum were obtained from Kansas City Biological Company, L-glutamine from Grand Island Biological Company, and gentamicin (Schering) from Microbiological Associates. N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Hepes), thymidine, and adenosine-5'-triphosphate (ATP) were obtained from Calbiochem. Horseradish peroxidase (Type II) (HRP), polylysine, hexokinase, O-diansidine, bovine serum albumin Fraction V (BSA), and buffered firefly lantern extract (Cat. No. FLE-50) were bought from Sigma; Permablend I from Packard; 70% glutaraldehyde from Ladd Research, carbonyl cyanide m-chlorophenylhydrozone (CCCP) from Aldrich; Paper Mulberry and Lycopodium spores from Coulter Electronics; [^{14}C(U)]sucrose, [^{14}C-carboxyl]inulin, [^{3}H(G)]inulin, [^{3}H-methoxy]inulin, [1-^{3}H(N)]L-glucose, [1-^{3}H(N)]D-mannitol from New England Nuclear; ^{86}RbCl from ICN; and [^{14}C]leucine and [^{3}H]thymidine from Amersham-Searle. All other chemicals used were reagent grade.

Methods

Cell culture. HeLa S3 cells (Puck et al., 1956) were grown in S-EMEM (Eagle, 1959) supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 µg/ml gentamicin, and 20 mM Hepes, pH 7.4. Sodium bicarbonate concentration was reduced to 2.5 mM (0.1 of the normal

amount for S-EMEM). The complete medium as described above is referred to as "growth medium." Suspension cultures were swirled at 110 c/min at 36°-38°C in screw-cap Erlenmeyer flasks. Attached cultures were maintained in Falcon or Corning T-75 flasks in air at 36°-38°C. Routine testing (Schneider et al., 1974) showed cultures to be free of mycoplasma contamination.

Growth of the suspension cultures was routinely monitored with a Coulter Counter (Model B). Cell number increased exponentially from 10^5 cells/ml to 8×10^5 cells/ml. The generation time was 26-30 hr. Cell size was determined with a Coulter Channelyzer to be 1600-2000 μm^3.

Cell synchrony. Cells were synchronized by the double-thymidine block technique of Petersen and Anderson (1964). Cells were incubated in growth medium containing 2 mM thymidine for 15 hr. The cells were then centrifuged and resuspended for 9 hr in growth medium with no thymidine. Subsequent incubation in 2 mM thymidine was again for 15 hr. DNA synthesis begain 3-4 hr after release from the second thymidine block. Greater than 90% synchrony (Chapter VI) was achieved.

Assay for uptake of markers for pinocytosis.

1. Suspension cultures. The desired amount of suspension stock culture was centrifuged in an International PR-6 centrifuge (284 rotor) at 500 × g for 10 min in Bellco centrifuge bottles (Cat. No. 3046). The cells were resuspended either in growth medium containing the marker (Method I) or in growth medium to which marker was subsequently added

(Method II). Pinocytic rate is independent of cell density during the uptake assay between 4×10^5 and 2×10^6 cells/ml. However, assays were routinely done at $4\text{-}8 \times 10^5$ cell/ml so that the cells would continue to grow exponentially during the assay. Radioactive markers were used at concentrations of 0.5-1 μCi/ml, and horseradish peroxidase (HRP) at 0.5 mg/ml. Triplicate ("0 time") samples to be substracted as blanks were harvested immediately as follows: $1\text{-}2 \times 10^6$ cells (usually 2-5 ml) per sample were pipetted into disposable, plastic, conical 15 ml Corning centrifuge tubes (Cat. No. 25300) containing 8-10 ml 37°C growth medium. The samples were centrifuged at $1000 \times g$ for 2 min in an International PR-6 centrifuge (rotor 253). The medium was decanted and the tubes inverted onto a paper towel. The tubes were then wiped out with a tissue; care was taken to avoid the cells in the tip of the tube. The samples were then washed with 5 ml of 37°C growth medium after very gentle resuspension, centrifuged as described above, and the excess medium again wiped out with a tissue. The remaining samples were harvested in this manner at the desired time after marker addition. This method for harvesting samples was adequate for removal of all the extracellular marker. It was found that assay in growth medium instead of buffered salt solution minimized cell loss during sampling. Using 37°C growth medium instead of 4°C medium facilitated thorough washing and also minimized cell losses.

a. Assay for radioactivity. If the samples harvested as described above were to be assayed for radioactivity, 0.5 ml of 0.1 M NaOH was

added to each sample to solubilize the cells. Samples were then removed with a Pasteur pipette into glass scintillation vials. Each tube was then rinsed with an additional 0.5 ml 0.1 M NaOH, pooled with the first lysate, and neutralized with 0.1 ml 1 M HCl. Ten ml of scintillation cocktail (composed of 2 ℓ toluene, 1 ℓ Triton X-100, and 16.5 g Permablend I) was added to each sample. The samples were assayed for radioactivity in a Nuclear Chicago Mark I scintillation counter.

b. HRP assay. Cell samples, harvested as described above, were sonicated on ice briefly with a Branson probe type sonicator (W-185-C) in 0.5-1.0 ml of 0.05 M sodium phosphate buffer, pH 5.0. The assays for HRP were then carried out as described by Steinman and Cohn (1972). Final concentrations in the reaction mixture were: 0.26 mM O-diansidine (substrate), 0.09 M H_2O_2, 0.05 M sodium phosphate buffer, pH 5.0, and approximately 100 μg of protein from the sonicated cell samples described above (equivalent to about 4×10^5 cells). The reaction was carried out at 25°C and monitored with a Gilford 2400 spectrophotometer and recorder at 460 nm. Only the portion of the curve which increased linearly with time was used to determine the reaction rate.

2. Attached cultures. The growth medium on replicate 60 mm plates with $1\text{-}2 \times 10^6$ cells/plate was aspirated and replaced with fresh growth medium containing the marker at the final concentrations indicated for assay in suspension culture. The medium on replicate plates, to be substracted as blanks, was immediately aspirated, and the plates were rinsed gently 3 times with PBS (0.16 M NaCl; 0.01 M sodium phosphate

buffer, pH 7.4). Cells were removed by lysis in 1.0 ml 0.1 M NaOH, neutralized with HCl, and assayed for radioactivity as described above for the suspension cultures. HRP assays were not done on attached cells.

Calculation of pinocytic rates. Good markers for nonadsorptive pinocytosis do not bind to the cells and are not taken up by specific transport systems (Chapter I). Therefore, quantitation of their uptake is a measure of medium uptake by the cells. Pinocytic rates were calculated as follows:

$$\frac{\frac{P-B}{C}}{M}$$

where P = uncorrected amount of pinocytic marker taken up; B = the amount of marker taken up at "0 time" after marker addition (see above); C = cell number per sample; M = amount of marker per pl of medium. Corrected results were then plotted as a function of time and pinocytic rates were graphically determined. Rates are expressed as pl/cell/hr.

ATP assay. The luciferase assay of Strehler (1974) was used. Lyophilized firefly lantern extract, which contains luciferin and luciferase, was hydrated with 5 ml water according to the instructions on the container, and the insoluble precipitate was removed by centrifugation. Removal of this precipitate did not cause loss of activity. About 8×10^6 cells, collected by centrifugation and immediately frozen, were lysed in 3.5 ml hot water and incubated at

80°-90°C for 15-30 min to inactivate cellular ATPases. The lysed cells were then mixed with 0.5 ml of luciferin-luciferase solution. Fluorescence, produced by the hydrolysis of ATP, was monitored in a Perkin-Elmer fluorometer (Model 204) with the excitation lamp off and the analyzer wavelength set at 0 to collect all light. Under the assay conditions used, fluorescence decreases linearly with time for at least 30 min.

DNA and protein synthesis determinations. Cell samples were incubated at 37°C for 15 or 30 min in growth medium with either [^{3}H]thymidine (2 μCi/ml) for measurement of DNA synthesis or [^{14}C]leucine (0.5 μCi/ml) for protein synthesis determination. Replicate samples were centrifuged at 1000 × g for 2 min. The pellets were then treated briefly with cold 10% trichloroacetic acid (TCA), centrifuged as described above, and washed once with 10% TCA, then once with ethanol. The samples were then treated as described above for assay of radioactivity. When both DNA and protein synthesis were measured in the same experiment, the two labeled compounds were added simultaneously in a ratio of 4:1 (^{3}H:^{14}C).

$^{86}Rb^{+}$ efflux assay. HeLa suspension cells were incubated at 37°C for 3 hr in growth medium containing 0.3 μCi/ml ^{86}RbCl. The cells were then centrifuged at 1000 × g for 2 min and resuspended in fresh growth medium with no ^{86}RbCl. Replicate samples were harvested by centrifugation at 1000 × g for 2 min at various times after removal of the label. The excess medium was removed with a tissue, and the pellets were assayed for radioactivity as described above.

N-acetyl-β-glucosaminidase assay. The assay mixture consisted of 5 mM p-nitrophenyl-N-acetyl-β-glucosamide (substrate); 100 mM sodium acetate buffer, pH 5.0; and enzyme (an aliquot of the 22,000 × g pellet described below under cell fractionation) in a final volume of 0.4 ml. Samples were assayed at 37°C usually for 20 min. The reaction was stopped by the addition of 0.8 ml of 0.5 M sodium glycine buffer, pH 10.4. Free p-nitrophenol was detected by reading the absorbance at 410 nm with a Gilford spectrophotometer. Under the assay conditions used, the reaction was linear with enzyme concentration or with time up to an A_{410} of 1.7 (approximately 4% conversion).

Purity determination of [^{3}H]L-glucose by hexokinase assay. D-glucose is rapidly phosphorylated to glucose-6-phosphate by hexokinase. L-glucose on the other hand, is not a substrate for hexokinase. I made use of the latter fact in determining whether [^{3}H]L-glucose purchased from New England Nuclear was free of contamination by D-glucose. Hexokinase was assayed by a modification of the procedure of Kletzien and Perdue (1973). The assay mixture contained 0.01 M Tris, pH 7.4; 1 μCi/ml [^{3}H]L-glucose (55 nM) or [^{3}H]D-glucose (1 μM); 0.1 unit of Sigma hexokinase suspended in 3.2 M $(NH_4)_2SO_4$, 0.25 mM ethylenediamine tetraacetic acid (EDTA), and 2.5 mM potassium phosphate, pH 7.0; 1.8 mM $MgCl_2$; and 1 mM ATP in a final volume of 0.25 ml. The samples were incubated at 37°C for 30 min and chilled to stop the reaction. Under these conditions, 75% conversion of D-glucose to D-glucose-6-phosphate occurred. An aliquot of each sample was then streaked onto a 3.5 cm wide Whatman 3MM paper strip and run in

95% EtOH: 1 M ammonium acetate, pH 5.0 (70:30) (Renner et al., 1972) for 8 hrs at room temperature. To assay for radioactivity, the chromatograms were cut into 1 cm wide pieces starting 0.5 cm behind the origin. Each 1 cm piece was placed in a glass scintillation vial, and the radioactivity was eluted into 1 ml H_2O. Scintillation cocktail (10 ml) was added and the samples counted as described above. The R_f glucose = 0.70 and R_f glucose-6-phosphate = 0.46. [^{3}H]L-glucose (Lot No. 919-050 from New England Nuclear) contained < 0.1% D-glucose.

[^{14}C]sucrose purification and chromatography of cell extracts. [^{14}C]sucrose from New England Nuclear contains about 0.5% glucose and fructose. These monosaccharides are taken up by specific transport systems much more rapidly than sucrose is taken up by micropinocytosis, and, therefore, [^{14}C]sucrose was purified by paper chromatography. About 300 µCi was streaked onto a 22-23 cm wide piece of Whatman 3MM paper and run for 30-50 hrs at 25°C in water-saturated butanol:trichloroethylene: 95% ethanol (6:2:2) (Chan and Cain, 1966). The chromatogram was dried well and then exposed to Kodak no-screen medical X-ray film for 2-24 hr. The film was then developed for 7 min in Kodak rapid X-ray developer and fixed for 6 min in Kodak X-ray fixer. The streak corresponding to sucrose was matched to the chromatogram, cut out, and stored dry at -20°C. R_{Gluc}sucrose = 0.45. R_{Gluc}fructose = 1.40 (Chapter IV).

Cell sonicates and extracts of cells which had taken up sucrose for different amounts of time were also chromatographed to determine whether sucrose is metabolized by the cells (Chapter IV). Cell samples harvested

as described above from sucrose-containing medium were sonicated in a small amount of water. The sonicate or the sonicate supernatant after centrifugation to remove cell debris was streaked onto 3.5 cm wide strips of Whatman 3MM paper, run in the solvent system described above, and assayed for radioactivity as described for [^{3}H]L-glucose purity determination.

[^{3}H]D-mannitol purification. Purification was carried out as described for [^{14}C]sucrose, except the chromatogram was run as described for [^{3}H]L-glucose purity determination. An exposure time for the autoradiogram of 1 wk was required since ^{3}H is a less energetic isotope than is ^{14}C. The R_f of mannitol = 0.73 and that of the major contaminant (10%) = 0.5.

Inulin purification. Contamination of inulin with its hydrolysis product fructose might cause artifactually large estimations of micropinocytic rates due to uptake of fructose. Therefore, radioactive inulin was run on a gel filtration column from which only the high molecular weight radioactivity was collected and used for micropinocytic rate measurements. A 0.8 × 16 cm column of Sephadex G-25 fine equilibrated with 0.01 M sodium phosphate buffer, pH 7.4 was used (Cohen, 1969). The sample was applied to the column in a small volume (0.2-1.0 ml) of buffer. The column was run at its maximum flow rate of 0.3 ml/min, and 20 drop (0.5 ml) fractions were collected. The void volume measured with blue dextran was about 5 ml (200 drops). The major peak of radioactivity eluted in fractions 14-25, with some trailing (smaller molecular weight

polymers) in later fractions (Chapter IV). Fractions 14-20, containing molecules with a minimum molecular weight of 2000, were pooled and used for measurements of micropinocytic rates.

Cell fractionation. Cells (usually about 2×10^7) were homogenized in a 7 ml Dounce homogenizer with a tight fitting B pestle in H medium (0.22 M mannitol; 0.07 M sucrose; 2 mM HEPES, pH adjusted to 7.4 with KOH) (Pedersen et al., 1977) containing either 0.05% (buffer 1) or 1% (buffer 2) BSA. The homogenate was centrifuged at 660 × g in an International PR-6 centrifuge (rotor #253) for 15 min to eliminate unlysed cells and nuclei (low-speed pellet). The supernatant (low speed) was then centrifuged at 22,000 × g for 30 min in a Sorvall RCB3 with an HB-4 rotor. The amounts of marker present in the low speed pellet, 22,000 × g pellet, and 22,000 × g supernatant were then determined by radioactive assay as described above.

Scanning electron microscopy (SEM). Progress in the development of a suitable method for SEM of HeLa suspension cells is discussed in detail in Chapter VII. Briefly, cells were fixed by mixing equal volumes of cell suspension in growth medium and 2% glutaraldehyde in S-EMEM (serum-free) at 37°C. The suspension was then poured into a plate containing several polylysine-coated microslides (1 cm^2) in the holder described in Chapter VII. The cells were fixed for 30-60 min, while settling onto the microslides. Ethanol was added dropwise to a final concentration of 10%. The glutaraldehyde-medium solution was gradually replaced by ethanol as described in Chapter VII. The microslides were critical point

dried from CO_2 in a Bomar SPC-1500 critical point dryer, coated with gold palladium in a Technics Hummer II specimen coater, and observed in an ETEC Autoscan scanning electron microscope.

CHAPTER III

KINETICS OF PINOCYTIC MARKER UPTAKE

In this section, I show that micropinocytosis, as defined by the experimental criteria given below, is linear with time, and that the rapid initial influx observed in my early assays of sucrose uptake was caused by mechanical manipulation of the cells and was not related to micropinocytosis. I demonstrate that sucrose, inulin, and HRP are taken up by HeLa S3 suspension cells at similar rates, which correspond well to micropinocytic rates observed with other cell systems (Table III-1). In contrast, L-glucose and D-mannitol, which are not specifically transported in other cell systems (Weber, 1973; Kletzien and Perdue, 1975; Dolberg et al., 1975, and Salter, 1977), are taken up at rates which are about tenfold greater than those for sucrose, inulin, or HRP. I conclude that the primary mode of influx for the above monosaccharides is diffusion, and, therefore, they cannot be used as markers for micropinocytosis. My data also demonstrate that, while low temperature markedly decreases micropinocytosis, this process is not affected by substantial depletion of cellular ATP or disruption of microfilaments by cytochalasin B. The two latter observations are the experimental criteria by which the pinocytic process measured in HeLa S3 suspension cells is characterized as micropinocytosis (Chapter I).

TABLE III-1

UPTAKE RATES OF SEVERAL COMMONLY USED PINOCYTIC MARKERS

Marker Molecule	Cell System	Pinocytic Rate pl/cell/hr	References
Soluble ferritin	Sarcoma 180	0.14	Petitpierre-Gabathuler et al., 1975
Sucrose	Chang liver cells	0.11	Wagner et al., 1971
HRP	L cells	0.035	Steinman et al., 1976
HRP	Macrophages	0.303	Steinman et al., 1976
Hemoglobin	Macrophages	0.10	Ehrenreich and Cohn, 1968
Polyvinylpyrrolidone	Rat yolk sac		Williams et al., 1975a; 1975b
Colloidal gold	Mouse macrophages	3.9	Davies et al., 1973a; 1975b

Rapid Initial Uptake of Sucrose

My initial assays of sucrose uptake using Method I (Chapter II) showed two-component kinetics. The first component was a very rapid uptake which was complete in samples harvested at "0 time." The second component was a slow uptake, which increased linearly with time for 10 hr (Fig. III-1). The average magnitude of the rapid uptake from several experiments was approximately equal to the average hourly uptake rate determined from the slow component in the same experiments and was, thus, a significant fraction of the total initial uptake. The similarity of the micropinocytic rate calculated from the slow uptake component and rates observed in other cell systems (Table III-1) suggested that the rapid sucrose uptake was unrelated to micropinocytosis. In other cell systems, the rapid uptake is evident from linear uptake curves with nonzero y-intercepts but has not been discussed (Wagner et al., 1971; Becker and Ashwood-Smith, 1973).

The average rapid sucrose influx from several experiments is 0.056± .007 S.E.M. pl/cell (n = 7). Table III-2 is a composite of different experimental conditions used to find the source of the rapid sucrose uptake. First, the rapid influx is not due to residual extracellular marker, since washing "0 time" samples three times instead of one time (Chapter II) did not decrease the amount of cell-associated radioactivity. Second, the addition of 10 mM nonradioactive sucrose to the uptake medium did not decrease the magnitude of the rapid uptake, and, thus, the rapid uptake is probably not due to specific binding of sucrose to the cell membrane. Third, a fourfold increase in the glucose concentration of

Fig. III-1. Comparison of sucrose and L-glucose uptake in HeLa suspension cells.

Triplicate alliquots of cell suspension were harvested at the indicated times by micropinocytic assay Method II (Chapter II). Error bars here and in subsequent figures in this chapter are ± one S.D., ●-●, sucrose; 0-0, L-glucose.

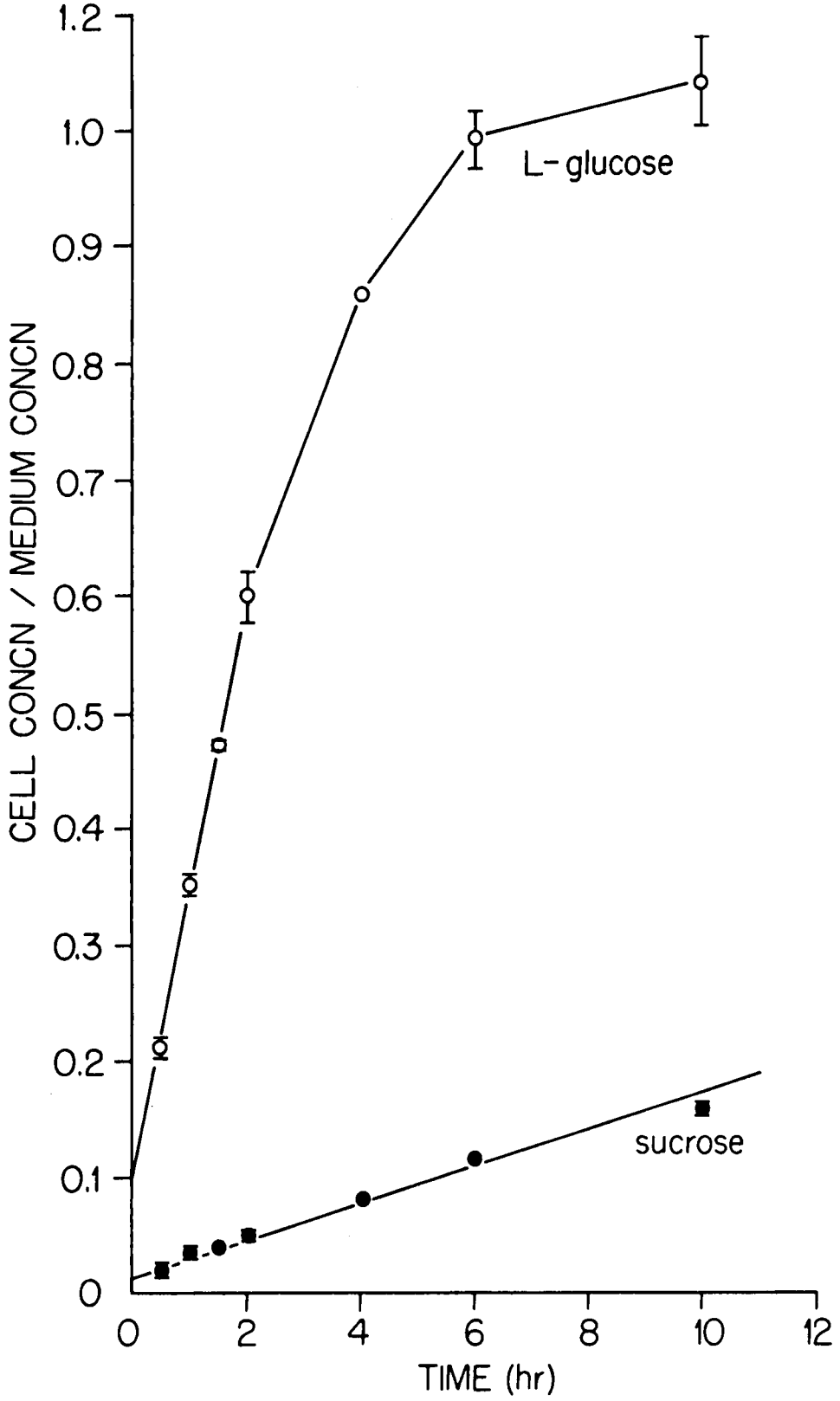

1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CELL CONCN / MEDIUM CONCN
L-glucose
sucrose
0
2
4
6
8
10
12
TIME (hr)

Fig. III-1

TABLE III-2

EFFECT OF SEVERAL EXPERIMENTAL TREATMENTS ON THE RAPID SUCROSE INFLUX[a]

Experimental Treatment	Rapid Sucrose Influx, Percent Control
Tracer [^{14}C]sucrose (2.3×10^{-6} M) (Method I control)	100
Three washes after harvesting	153
10 mM nonradioactive sucrose	97
20 mM glucose	100
Purification of [^{14}C]sucrose	100
Heat-killed cells	0
Attached cells	0
Recovery after centrifugation and pipetting	
0-5 min	0
20 min	0
40 min	0
No serum	184
5 mM 2-dGlc[b]; ~0.25 mM D-glucose; 10 mM sodium azide	200
Polyamino acids	
2×10^{-8} M poly-D-lysine	233
6×10^{-8} M poly D-lysine	700
2×10^{-8} M poly-L-ornithine	155
6×10^{-8} M poly-L-ornithine	444

[a]Control rapid influx was assayed by Method I (Chapter II). Samples were harvested at "0 time" only. Experimentals were assayed by Method I except for the designated experimental treatment. The results are expressed as percent of control rapid influx and are the average of triplicate determinations.

[b]2-deoxyglucose.

the assay medium or purification of the [^{14}C]sucrose prior to its use in the uptake assay (Chapter II) did not eliminate the fast component, indicating that it did not represent the rapid, specific transport of contaminating glucose or fructose present in the [^{14}C]sucrose. Finally, leakage of sucrose into a small number of nonviable cells was not the source of the rapid uptake component. Fig. III-2 shows that the magnitude of the rapid influx component increased linearly with the number of viable cells per sample, did not occur at all in 100% heat-killed cells, and is, therefore, a property of viable cells. Apparently, sucrose, which permeates nonviable cells freely, was rapidly removed by the harvesting and washing technique described in Chapter II and, thus, was not detected as cell-associated radioactivity.

When the rates of sucrose uptake in attached and suspension HeLa cells were compared, no rapid sucrose uptake was noted in the attached cells. Two differences between the attached cell and suspension cell assays, which might relate to the rapid uptake were: (1) the attached cultures were washed more extensively after assay than were the suspension cultures, and (2) at the beginning of the assay, the suspension cultures were centrifuged and resuspended by pipetting in [^{14}C]sucrose-containing medium (micropinocytosis assay Method I, Chapter II). As noted above, multiple washes of "0 time" samples from suspension uptake assays did not reduce the rapid sucrose influx and, therefore, the first difference between attached cell and suspension cell assays was not related to the rapid sucrose uptake. The second difference was investigated by comparing micropinocytic assay Methods I and II (Chapter II). Two samples

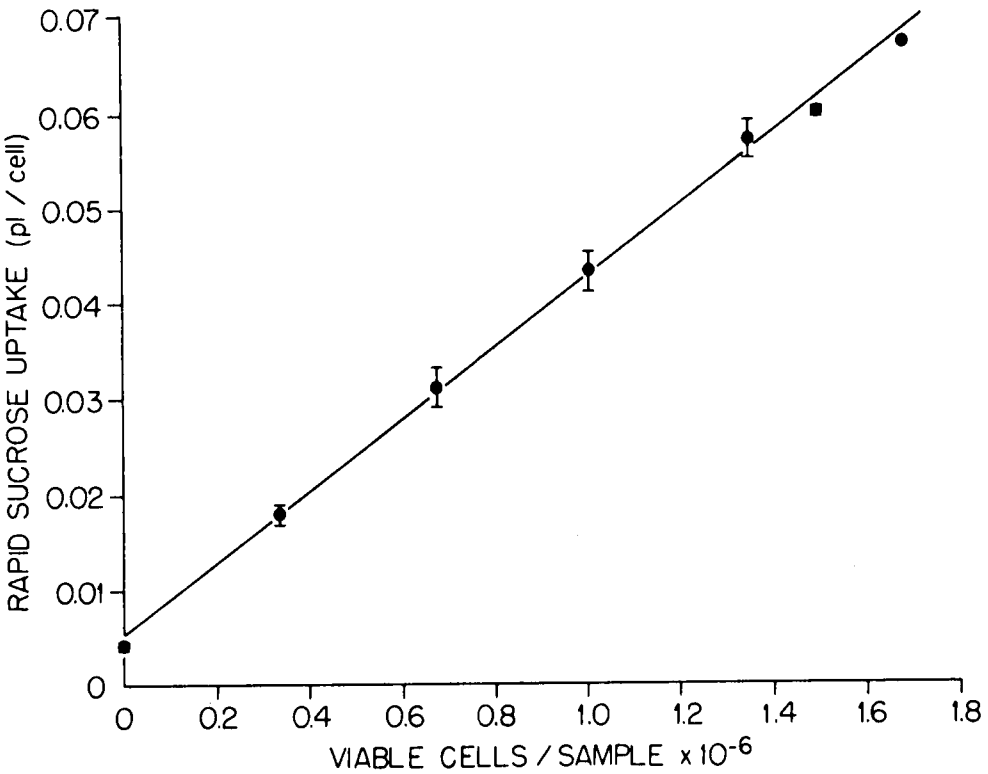


Fig. III-2. Dependence of the rapid sucrose influx on cell viability.

HeLa suspension cells were killed by heating to 65°C for 30 min. Sample flasks were prepared by mixing viable and heat-killed cells in different proportions to yield a constant final concentration of cells in each sample flask. Triplicate aliquots were harvested from each sample flask at zero time only by micropinocytic assay Method I (Chapter II). Uptake is expressed as pℓ of medium taken up per cell. The total number of cells per sample (viable and nonviable) was used for the latter calculation.

of the same stock culture of HeLa suspension cells were harvested by centrifugation. Half of the cells were resuspended in [^{14}C]sucrose-containing growth medium, and samples were harvested immediately as described in Chapter II (Method I). The remaining cells were resuspended in fresh nonradioactive growth medium and returned to normal growth conditions (Method II). At several times after resuspension, a small amount of concentrated [^{14}C]sucrose solution was added to aliquots of culture, and samples were harvested immediately. Cells assayed by Method II showed no rapid influx regardless of recovery time after centrifugation and resuspension in fresh medium (Table III-2, p. 21).

The rapid influx observed with assay of sucrose uptake also occurred when assay Method I was used for measurements on inulin and HRP uptake. Thus, this phenomenon is not due to specific properties of the sucrose molecule. A possible explanation for the rapid uptake is that mechanical injury caused by centrifugation and pipetting of the cells stretches the membrane making "holes" or weak areas where marker molecules can diffuse into the cells. Immediate resealing of the "holes" after return of the cells to normal growth conditions may trap marker molecules inside the cell. Nonviable cells, on the other hand, cannot repair their membranes and, thus, cannot trap marker molecules. This hypothesis is supported by the observation that conditions which adversely affect cell growth and might induce membrane damage increased the magnitude of the rapid uptake. For example, assay in serum-free medium, in the presence of high concentrations of sodium azide (see also below), or in the presence of polyamino acids substantially increased the size of the rapid uptake component (Table III-2, p. 21).

Effects of positively charged polyamino acids on micropinocytic rates in HeLa cells were of interest, since stimulation of this process by polylysine and polyornithine in Ehrlich ascites and sarcoma 180 cells has been reported previously (Ryser and Hancock, 1965; Ryser, 1967a, 1967b). The above polyamino acids seemed to substantially stimulate micropinocytosis measured by sucrose uptake. Later, however, it became apparent that this stimulation was due to an enhanced rapid uptake component and not to enhanced micropinocytosis. This increase in the rapid influx was directly proportional to polyamino acid concentration, polylysine being more effective than polyornithine (Table III-2, p. 21). The largest increase occurred at a concentration (6×10^{-8} M) which severely impairs cell growth, decreases protein synthesis, and increases cell permeability to ions as measured by $^{86}Rb^{+}$ efflux rates (Table III-3). Other investigators have found that stimulation by polyamino acids did not occur at nontoxic concentrations (Wallace and Ho, 1972; Seljelid et al., 1973).

In summary, the rapid influx, which can make up a large fraction of the total influx measured by Method I, is not related to micropinocytosis. The data suggest that it is a result of membrane damage and immediate repair, which occurs when viable cells are centrifuged and pipetted in marker-containing medium.

Comparison of Uptake of Micropinocytic Markers

Since it was desirable to eliminate the rapid initial influx of markers, uptake assay Method II was used in subsequent experiments

TABLE III-3

EFFECT OF POLYAMINO ACIDS ON SEVERAL PARAMETERS OF VIABLE CELLS[a]

Parameter Measured	Percent of Control
Rate constant for $^{86}Rb^+$ efflux ($1/T_{37}$)	250
[^{3}H]thymidine incorporation	91
[^{3}H]leucine incorporation	71
Growth rate	
2×10^{-8} M poly-D-lysine	85
6×10^{-8} M poly-D-lysine	0
2×10^{-8} M poly-L-ornithine	100
6×10^{-8} M poly-L-ornithine	50

[a]Protein synthesis, DNA synthesis, and $^{86}Rb^+$ efflux were measured at described in Chapter II after a 3 hr treatment with 6×10^{-8} M poly-D-lysine (experimentals). Growth experiments were carried out over a period of 3 days at the indicated polyamino acid concentrations. Results are expressed as percent of control with no polyamino acids.

except where indicated. Table III-4 gives average uptake rates for the markers tested. The rates of sucrose and inulin uptake were not statistically different and corresponded well to micropinocytic rates in other cell systems (Table III-1, p. 21). The HRP uptake rate was similar to that of sucrose and inulin. However, HRP is a less convenient marker in HeLa cells than the latter two molecules, since the HRP assay is more time consuming, less reproducible, and involves the use of a carcinogenic substrate. My data also indicate that there may be some nonspecific binding of HRP to HeLa cells and that measurements may be complicated by metabolism of HRP by lysosomal enzymes (Chapter IV). Binding of HRP to cells has also been observed in other systems (Graham and Karnovsky, 1966; Creemers and Jacques, 1971). Sucrose probably does not bind to cells, since the presence of 1000 fold excess of nonradioactive sucrose (10 m$\underline{M}$) does not change the rate of [^{14}C]sucrose uptake. Likewise, inulin does not appear to bind to HeLa cells because its uptake rate is similar to that of sucrose.

Sucrose, inulin, and HRP uptake rates seem to be good measures of micropinocytosis. Two lines of evidence suggest that simple diffusion accounts for only a very minor fraction of their uptakes. First, these markers are taken up very slowly and at similar rates even though their molecular weights range over two orders of magnitude (Table III-4). Second, the uptake rate of sucrose and inulin is reduced to the same extent (10%-15% of the 37°C control) by assay at 4°C, even though inulin is 15 times larger than sucrose.

On the other hand, L-glucose and D-mannitol, which are non-permeating monosaccharides in most cell systems, are taken up much

TABLE III-4

UPTAKE RATES OF PUTATIVE MICROPINOCYTIC MARKERS[a]

Marker	Average Uptake Rate, pl/cell/hr
Sucrose	0.050 ± 0.007 (n = 6)[b]
Inulin	0.043 ± 0.007 (n = 5)[b]
HRP	0.093[c]
L-glucose	0.840 (Exp. 1)[c]; 0.500 (Exp. 2)[c]
D-mannitol	1.170 (Exp. 1)[c]; 0.840 (Exp. 2)[c]
Ouabain, 2×10^{-8} M	0.737[c]

[a]Uptake was measured by micropinocytic assay Method II and rates calculated as described in Chapter II.

[b]Average rate ± S.E.M., n = number of experiments, triplicate determinations were done in each experiment.

[c]Average rate determined from triplicate samples in one experiment. Data for two separate experiments are given for L-glucose and D-mannitol.

faster than sucrose, inulin, or HRP. There are several lines of evidence which suggest that these sugars enter HeLa cells primarily by diffusion. Fig. III-1, p. 19, shows that the influx of L-glucose, calculated from the linear portion of the curve, was tenfold greater than that of sucrose and that the cellular L-glucose concentration equilibrated with the medium concentration after 6 hr. D-mannitol also showed rapid equilibration between cellular and medium concentrations (Fig. III-3). Although the equilibrium concentration attained by D-mannitol or L-glucose was constant, the rate at which equilibrium was reached varied from experiment to experiment. Uptake rate values for two experiments are given in Table III-4.

The data presented for L-glucose and D-mannitol uptake thus far could fit either a diffusion or a facilitated diffusion mechanism. However, uptake of [^{3}H]D-mannitol is not affected by the presence of 10 mM nonradioactive D-mannitol and, therefore, facilitated diffusion, which involves binding of the substrate to a carrier, is an unlikely mode of uptake. I conclude that L-glucose and D-mannitol enter HeLa cells primarily by diffusion, that the permeability of the membrane to these monosaccharides varies from experiment to experiment, and, most important, that these sugars cannot be used as markers for micropinocytosis in HeLa cells.

Large errors in micropinocytic measurements (fluid uptake) can also be introduced by using a marker which binds to the cell membrane and, thus, effectively concentrates marker onto the cell surface from the medium. As demonstrated above, sucrose and inulin, which do not bind to

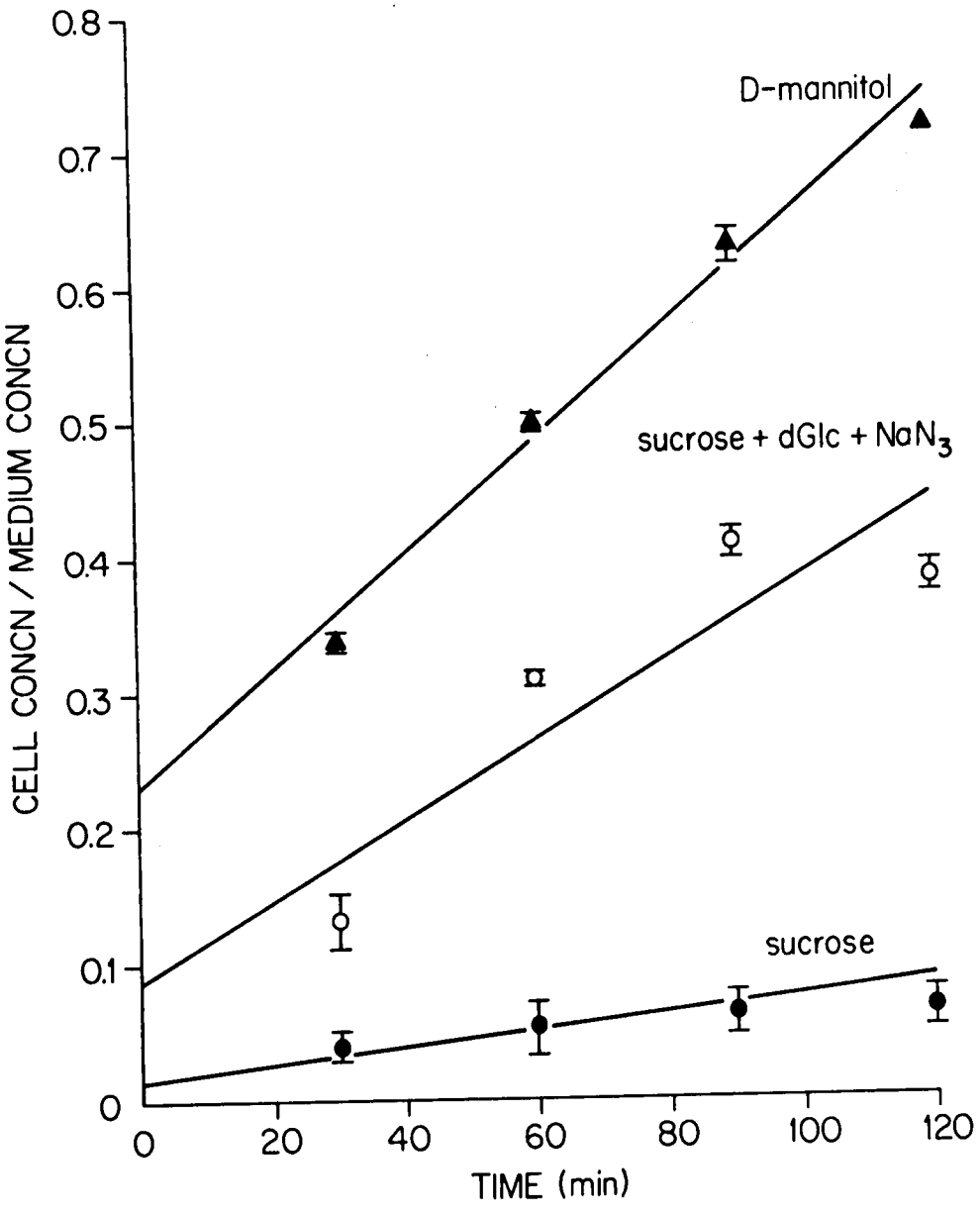


Fig. III-3. A comparison of D-mannitol and sucrose uptake in the presence and absence of ATP depleting agents.

HeLa suspension cells were preincubated in glucose-free growth medium (controls) or glucose-free growth medium plus 5 mM 2-dGlc and 10 mM sodium azide (NaN_3) (experimentals) for 30 min. Triplicate aliquots from each sample flask were harvested at the indicated times and assayed for micropinocytosis by Method II (Chapter II). Sucrose uptake measured in a parallel control with 5 mM glucose added (normal S-EMEM glucose concentration) did not differ from the low-glucose control described above. ●, sucrose (low-glucose) control; 0, sucrose + 2-dGlc + NaN_3; ▲, D-mannitol (low-glucose) control.

HeLa cells, show linear uptake kinetics. On the other hand, ouabain, a molecule which binds specifically to the plasma membrane Na^+, K^+ ATPase in HeLa cells (Baker and Willis, 1972; Lamb and McCall, 1972; and Cook et al., 1975), shows two-component kinetics of uptake (Fig. III-4). The concentration (2×10^{-8}M) used in this experiment produces only half saturation (5×10^5 sites/cell) of the available binding sites and is nontoxic to the cells. Component 1 (C_1) is specific binding of ouabain to the Na^+, K^+ ATPase, and component 2 (C_2) is probably adsorptive macropinocytosis (Chapter I and below). If binding is not taken into account and the pinocytic rate for [^{3}H] ouabain is calculated from the slope of component 2 by the method described in Chapter II, the uptake rate is an order of magnitude larger than that of sucrose, inulin, or HRP (Table III-4, p. 28), and the total ouabain space after 50 hrs is 120 pl/cell. The latter value is 60 times the cell volume, and is a prima facie argument for binding. The adsorptive macropinocytic rate is more accurately expressed as the time required to pinocytose the total number of bound sites (in this experiment: 5×10^5). This value equals the time required for the uptake of one complete membrane surface area (one membrane equivalent), assuming random distribution of ouabain-binding sites and random sites for pinocytosis. In the experiment described in Fig. III-4, the adsorptive macropinocytic rate is one membrane equivalent/15 hr, a value which agrees fairly well with previously determined rates of Na^+, K^+ ATPase turnover (Cook et al., 1976). The long term linearity and large concentration of medium ouabain by the cells indicate that, unlike L-glucose and D-mannitol

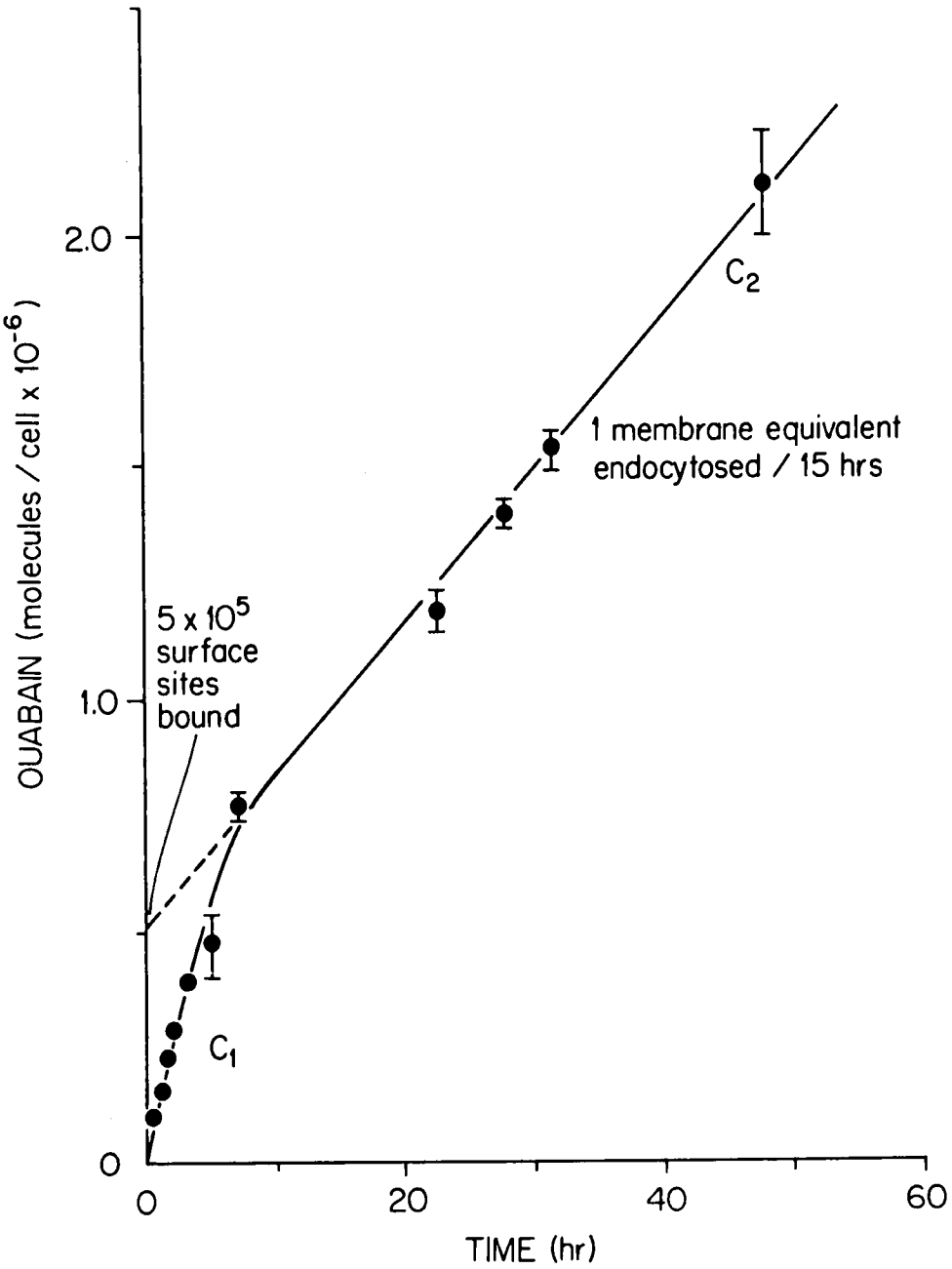


Fig. III-4. Long term uptake of ouabain in HeLa suspension cells.

Triplicate determinations of [^{3}H]ouabain (2×10^{-8} M) uptake by micropinocytic assay Method II (Chapter II) were done at the times indicated on the abscissa. The total number of molecules bound to the cells was determined by extrapolation of component 2 (C_2) to the ordinate. One membrane equivalent equals the number of bound surface sites (5×10^5 sites/cell for this experiment) (see text).

uptake, ouabain is not taken up primarily by diffusion. Cell fractionation experiments showing codistribution of internalized ouabain and lysosomal enzyme markers (Cook and Brake, 1978; Chapter IV below) indicate that adsorptive macropinocytosis is a likely mode of uptake.

In summary, sucrose and inulin are good markers for nonadsorptive pinocytosis in HeLa suspension cells by the criteria listed in Chapter I. These molecules are not metabolized (Chapter IV). They do not bind to the plasma membrane or diffuse readily into the cells. They are nontoxic and their uptakes are easily assayed. Sucrose is more stable than inulin, but both are adequately stable. Although large amounts of sucrose stimulate lysosomal enzymes in some systems (Fell and Dingle, 1966; Benassi, 1968; and Ricketts, 1971), sucrose does not stimulate its own pinocytic uptake (Nyberg and Dingle, 1970; Bernacki and Bosmann, 1971; and Wagner et al., 1971). HRP is taken up at a similar rate or possibly slightly faster than inulin or sucrose, but use of HRP as a marker is complicated by nonspecific binding to the cell surface and possible metabolism by lysosomal enzymes. Two other markers tested, L-glucose and D-mannitol, diffuse into the cell rapidly and, thus, cannot be used as markers. Ouabain, which binds to cells, can only be used as a marker for adsorptive macropinocytosis, a process which may not be the same for different specifically bound molecules.

Effects of ATP Depletion, Microfilament Disruption, and Cold

My early experiments using a reduced medium glucose concentration with added 2-deoxyglucose (2-dGlc) and sodium azide (NaN_3) to deplete

cells of ATP gave unexpected results. Fig. III-3, p. 30, shows that sucrose uptake was increased by the above combination of inhibitors to a level approaching the diffusional uptake of D-mannitol. D-mannitol uptake was not affected by the inhibitors (data not shown). Several attempts to find the concentrations of 2-dGlc and NaN_3 that deplete cellular ATP without permeabilizing the cells were unsuccessful. When CCCP, an uncoupler of oxidative phosphorylation, was used instead of NaN_3 in combination with 2-dGlc, ATP was depleted to 25% of its original concentration. However, there was no effect on micropinocytic rates measured by sucrose or inulin uptake (Table III-5). There was also no cell permeabilization with CCCP. Table III-5 also shows that these uptake rates were not inhibited by cytochalasin B but were reduced to 10%-15% of the 37°C control rate when assayed at 4°C.

In contrast, uptake of pulse-bound ouabain by adsorptive macropinocytosis was inhibited by ATP depletion with NaN_3 and 2-dGlc (Cook et al., 1976). Failure to detect permeabilization of the cells by NaN_3 as in the case of sucrose uptake measurements probably resulted from the bound state of the [^{3}H]ouabain and the absence of free [^{3}H]ouabain in the medium when the inhibitors were present. Adsorptive macropinocytosis of ouabain did not occur at low temperature, but I have not tested for inhibition by cytochalasin B.

There is some disagreement about dependence of endocytic processes on cellular ATP levels and intact microfilaments. Phagocytosis definitely requires energy and both cytoskeletal elements, i.e. microtubules and microfilaments (Casley-Smith, 1969; Stossel et al.,

TABLE III-5

EFFECT OF ATP DEPLETION, CYTOCHALASIN B, AND COLD ON MICROPINOCYTOSIS OF SUCROSE AND INULIN[a]

Treatment	Percent of Control Micropinocytic Rate	
	Sucrose Uptake	Inulin Uptake
ATP Depletion	109	100
Cytochalasin B		
6-7 μg/ml	98	N.D.
20 μg/ml	75	100
Cold (4°C)	12-14	10-15
Ethanol	102	100

[a]Uptake was measured by assay Method II, and rates were calculated as described in Chapter II. Depletion of ATP to 25% of control levels was accomplished by a 15-30 min preincubation before label addition in glucose-deficient growth medium with 5 mM 2-dGlc and 2×10^{-7} M CCCP. Glucose was from the fetal calf serum only and was ~0.25 mM, final concentration. Cytochalasin B at the indicated concentrations was added to some samples 15-30 min prior to the assay. Ethanol (80 mM) controls were included, since both CCCP and cytochalasin B stocks are made up in ethanol. Uptake in the cold (~4°C) was carried out in ice on a rotating table. Results are expressed as percent of control uptake rate in normal medium at 37°C.

1972; Wills et al., 1972; Davies et al., 1973c; Allison and Davies, 1974). However, requirements for pinocytosis are not so clear-cut. Some investigators report sensitivity of pinocytosis to ATP depletion (Cohn, 1966; Wagner et al., 1971; Poste and Papahadjopoulos, 1976) and cytochalasin B (Wagner et al., 1971; Poste and Papahadjopoulos, 1976), while others find no such sensitivity of pinocytic uptake, at least into small vesicles (Jennings and Florey, 1967; Casley-Smith, 1969; Petitpierre-Gabathuler and Ryser, 1975). Casley-Smith (1969) has shown that ferritin, a nonbinding marker for pinocytosis in macrophages, is taken up into a wide range of vesicle sizes and that only ferritin uptake into large vesicles is inhibited by ATP depletion. It has also been demonstrated that sensitivity of pinocytosis to cytochalasin B shows a similar dependence on vesicle size (Wills et al., 1972; Davies et al., 1973c; Allison and Davies, 1974). Based on these observations, Allison and Davies (1974) subdivided the pinocytic process into the categories described in Chapter I: (1) macropinocytosis (0.3-2 μm diameter vesicles, energy- and microfilament-dependent), and (2) micropinocytosis (0.07-0.1 μm diameter vesicle, energy- and microfilament-independent). Since the data presented above indicate that inulin and sucrose uptakes in HeLa suspension cells are not dependent on cellular energy or intact microfilaments, these influxes were classified as micropinocytosis. The pinocytic uptake of ouabain, on the other hand, was classified as adsorptive macropinocytosis since ouabain binds to the plasma membrane, and its uptake is ATP-dependent.

CHAPTER IV

INTRACELLULAR LOCALIZATION AND METABOLISM OF PINOCYTIC MARKERS

Molecules taken up by endocytosis are commonly localized in secondary lysosomes, which are formed by the fusion of endosomes with primary lysosomes (De Duve and Wattiaux, 1966; Holtzman, 1976 for review). Many molecules are then degraded by lysosomal enzymes (Gordon et al., 1965; De Duve and Wattiaux, 1966; Ehrenreich and Cohn, 1967, 1969; and Gabathuler and Ryser, 1969). It is shown in this chapter that when HeLa cells are fractionated as described in Chapter II, internalized ouabain codistributes with the lysosomal marker enzyme, N-acetyl-β-glucosaminidase. The data suggest that > 90% of the internalized ouabain and the lysosomal marker enzyme is associated with sedimentable cellular material. In contrast, not all intracellular sucrose and inulin are associated with sedimentable cellular material, presumably because these molecules are not membrane bound and can freely diffuse out of lysosomes damaged during cell homogenization.

Chromatography of extracts from cells which have taken up sucrose, inulin, or ouabain for long periods of time shows no metabolic breakdown products. This suggests that intralysosomal metabolism of these molecules does not occur. On the other hand, HRP appears to be degraded by HeLa cells fairly rapidly and is, thus, a less reliable marker for micropinocytosis than the former two molecules.

Cell Fractionation Studies

The cell fractionation method used yielded a final 22,000 × g pellet, which was free of nuclei and unlysed cells but contained plasma membrane, mitochondria, and probably endoplasmic reticulum in addition to lysosomes. Association of a tracer molecule with the 22,000 × g pellet suggests that: (1) the molecule is taken up from the medium into vesicles (pinosomes) and upon fractionation sediments with these vesicles; or (2) tracer molecules, which diffuse or are transported into the cell, become nonspecifically associated with the particulate fraction during cell homogenization. The data presented here fit explanation 1, although in one experiment there was some evidence that nonspecific adsorption or trapping might have occurred.

The pelletability of ouabain, the marker for adsorptive macropinocytosis, was first tested to determine whether it associated in a reproducible way with the 22,000 × g pellet. Fig. IV-1 shows that immediately after a pulse label with [^{3}H]ouabain, all ouabain pelleted at 22,000 × g regardless of the number of Dounce homogenization strokes in buffer 1. Cells that were returned to normal growth medium after pulse labeling and harvested for cell fractionation at different times post-label showed an inverse dependence of ouabain pelletability on the number of homogenization strokes. The dependence increased with increasing time post-label until a maximum dependence was obtained at about 24 hrs. The activity of the lysosomal marker enzyme, N-acetyl-β-glucosaminidase, had the same first order relationship to homogenization strokes as did ouabain 24 hrs post-label (Cook and Brake, 1978; Fig.

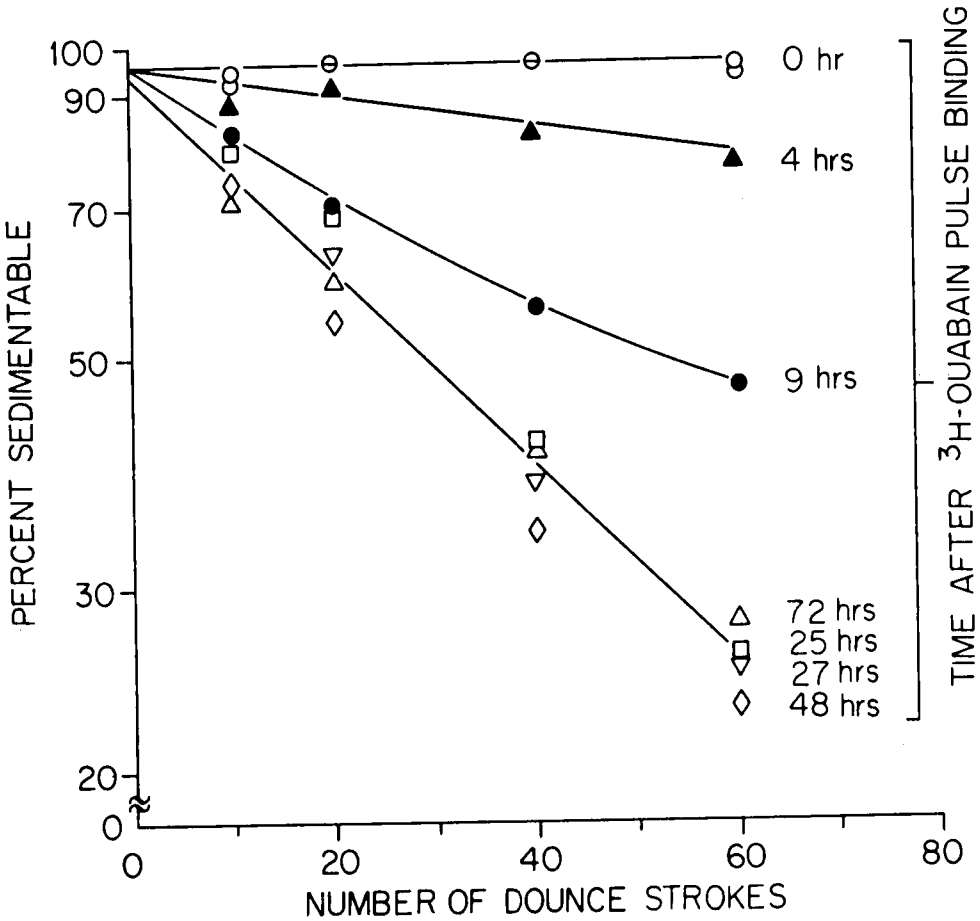


Fig. IV-1. Dependence of ouabain pelletability on number of homogenization strokes.

HeLa cells were pulse labeled for 15 min at 37°C with [^{3}H]ouabain at 2×10^{-7} M in growth medium with 1/10 the normal K^+ concentration (0.55 mM). A reduced K^+ concentration facilities binding, since K^+ is a competitive inhibitor of ouabain binding. The cells were then centrifuged and resuspended in ouabain-free growth medium. Cell fractionation was carried out in buffer 1 at the indicated times post label by the method described in Chapter II. The 22,000 × g pellet and supernatant were assayed for radioactivity as described in Chapter II.

IV-2). The above observations suggest that ouabain bound to the plasma membrane at "0 time" after the pulse label is insensitive to dissociation by homogenization. However, after internalization into pinosomes, some change in the binding of the ouabain to its plasma membrane site might occur which makes the ouabain or the ouabain-binding site complex more subject to leakage from lysosomes during homogenization. The ouabain may remain bound to some membrane fragment, since it does not leak as readily from lysosomes during homogenization as does sucrose, which is of comparable size (see below). The maximum dependence of ouabain pelletability on Dounce homogenization represents the time when virtually all of the ouabain has been internalized, hence the similar dependence of lysosomal marker enzyme pelletability on homogenization strokes (Fig. IV-2). Extrapolation of the curves to zero homogenization strokes, presumably a measure of in vivo conditions, shows > 90% pelletable ouabain regardless of time post-label. Table IV-1 shows that homogenization in buffer 2, which contains a 200-fold higher BSA concentration, gave less dependence of both internalized ouabain and N-acetyl-β-glucosaminidase pelletability on homogenization strokes. The explanation for this observation is unclear, but it might be that BSA either protects lysosomes against leakage during homogenization or that it causes nonspecific readsorption of homogenization-released ouabain to particulate material. Since the concentration of BSA used even in buffer 2 is only 1% (2×10^{-4} M), the BSA could not be protecting osmotically.

In contrast, pelletability of sucrose and inulin was independent of the number of homogenization strokes (Table IV-1). About 20% and

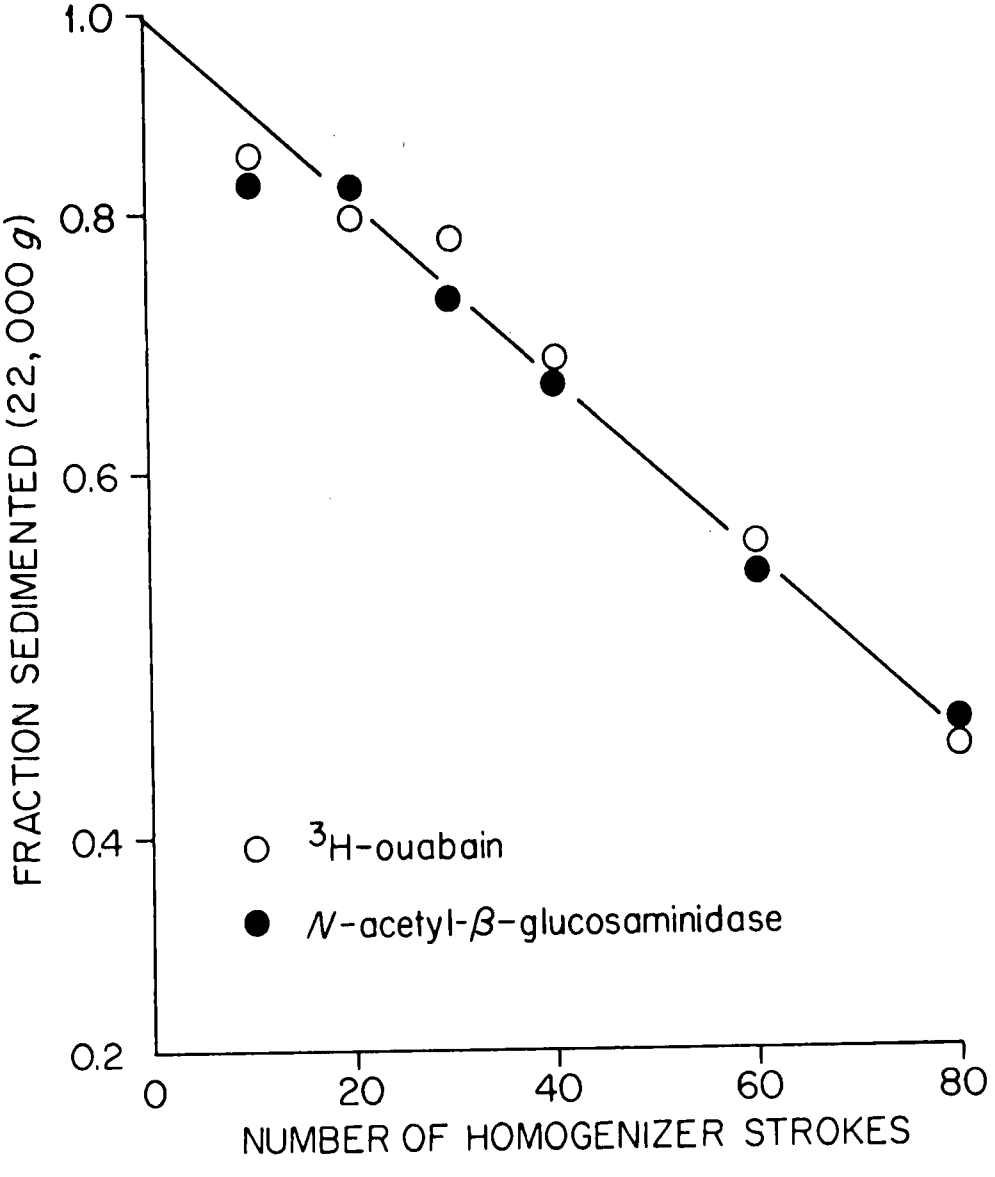


Fig. IV-2. Codistribution of internalized ouabain and N-acetyl-β-glucosaminidase activity.

HeLa cells were treated as described for Fig. IV-1. Cell fractionation was done 24 hr post label. The 22,000 × g pellet was assayed for radioactivity and N-acetyl-β-glucosaminidase activity as described in Chapter II. [^{3}H]ouabain, 0-0. N-acetyl-β-glucosaminidase, ●-●.

TABLE IV-1

CELLULAR DISTRIBUTION OF PINOCYTIC MARKERS[a]

Marker	Homogenization Buffer	Number Homogenization Strokes	Percent in High Speed Pellet
Ouabain	2[b]	10	75[c]
	2	20	88
	2	40	79
	2	60	68
N-acetyl-β-glucosaminidase	1	10	100
	1	20	88
	1	40	73
	1	60	56
N-acetyl-β-glucosaminidase	2	10	100
	2	20	93
	2	40	84
	2	60	75
Inulin	1	10	60
	1	20	51
	1	40	50
	1	60	51
Inulin	2	10	41[c]
	2	20	74
	2	40	55
	2	60	50
Sucrose	1	10	11
	1	20	17
	1	40	16
	1	60	13[d]
	1	75	25[d]
Sucrose	2 (Exp. 1)	5	3
	2	10	20
	2	20	42
	2	40	42
Sucrose	2 (Exp. 2)	10	6
	2	20	11
	2	40	16

[a]HeLa cells were homogenized in buffer 1 or 2 and fractionated as described in Chapter II except for two experiments in which the high speed centrifugation was 300,000 × g for 30 min instead of 22,000 × g for 30 min. Results are expressed as percent of the total low speed supernatant (see Chapter II) radioactivity found in the 22,000 × g or 300,000 × g pellet.

[b]See Fig. IV-1 for distribution after homogenization in buffer 1.

[c]Average of two experiments.

[d]Average of two experiments. High speed spin was 300,000 × g—30 min.

50% of the total intracellular sucrose and inulin respectively sedimented at 22,000 × g. A possible explanation for these observations is that: the unbound molecules, sucrose and inulin, diffuse out of lysosomes damaged by homogenization more readily than ouabain, which may remain membrane-bound intralysosomally.

Another complicating factor besides possible diffusion of sucrose and inulin out of lysosomes during homogenization might have been nonspecific adsorption of the above molecules to cell particulate material. In experiment 1, Table IV-1, pelletability of sucrose increased with the number of homogenization strokes. This observation, however, was not reproducible (Table IV-1, exp. 2) and, therefore, might have been due to nonspecific adsorption or trapping of sucrose. Difficulties encountered with the cell fractionation experiments, such as leakage of tracers from the lysosomes and nonspecific adsorption during homogenization point out the general problems encountered with cell fractionation experiments.

Metabolism of Micropinocytic and Adsorptive Macropinocytic Markers

Digestion of intralysosomal materials provides cells with nutrients and a means of detoxification of harmful substances. However, metabolism of micropinocytic marker molecules complicates interpretation of their uptake kinetics. For example, the HRP uptake curve becomes nonlinear before enough uptake has occurred to make highly reliable rate measurements. This is presumably because HRP is metabolized in HeLa suspension cells to a product, which is no longer a substrate for the HRP assay method described in Chapter II.

My early results from chromatographic experiments suggested that the micropinocytic marker sucrose might also be metabolized by HeLa suspension cells. About 30%-40% of the cell-associated radioactivity after 15-30 hr of [^{14}C]sucrose uptake did not migrate like authentic sucrose but stayed at the chromatogram origin. It was later found that water extracts of cell sonicates from which cell debris had been removed by centrifugation showed only about 5% of the total chromatogram [^{14}C] at the origin (Fig. IV-3). I concluded from this observation that sucrose is not metabolized by HeLa suspension cells and that the origin-associated material was an artifact of chromatography. These data are consistent with the observations of other investigators, who found that sucrose is a nonmetabolizable sugar (Cohn and Ehrenreich, 1969; Bernacki and Bosmann, 1971).

Likewise, inulin does not appear to be metabolized. Fig. IV-4, shows that cell-associated inulin (15 hr uptake) eluted from the G-25 column (see Chapter II) with blue dextran. This indicates that no breakdown of inulin to smaller molecular weight polymers or to fructose had occurred. The presence of cellular material on the column slightly shifted the normal elution profile of inulin to a higher apparent molecular weight. The cause of this shift is unknown. It is, however, unlikely that inulin was broken down by the cells and then metabolized into a high molecular weight substance. It is more likely that some nonspecific adsorption of inulin to cellular material occurred and that this aggregate eluted with blue dextran. Nonspecific adsorption of inulin metabolites to high molecular weight cell

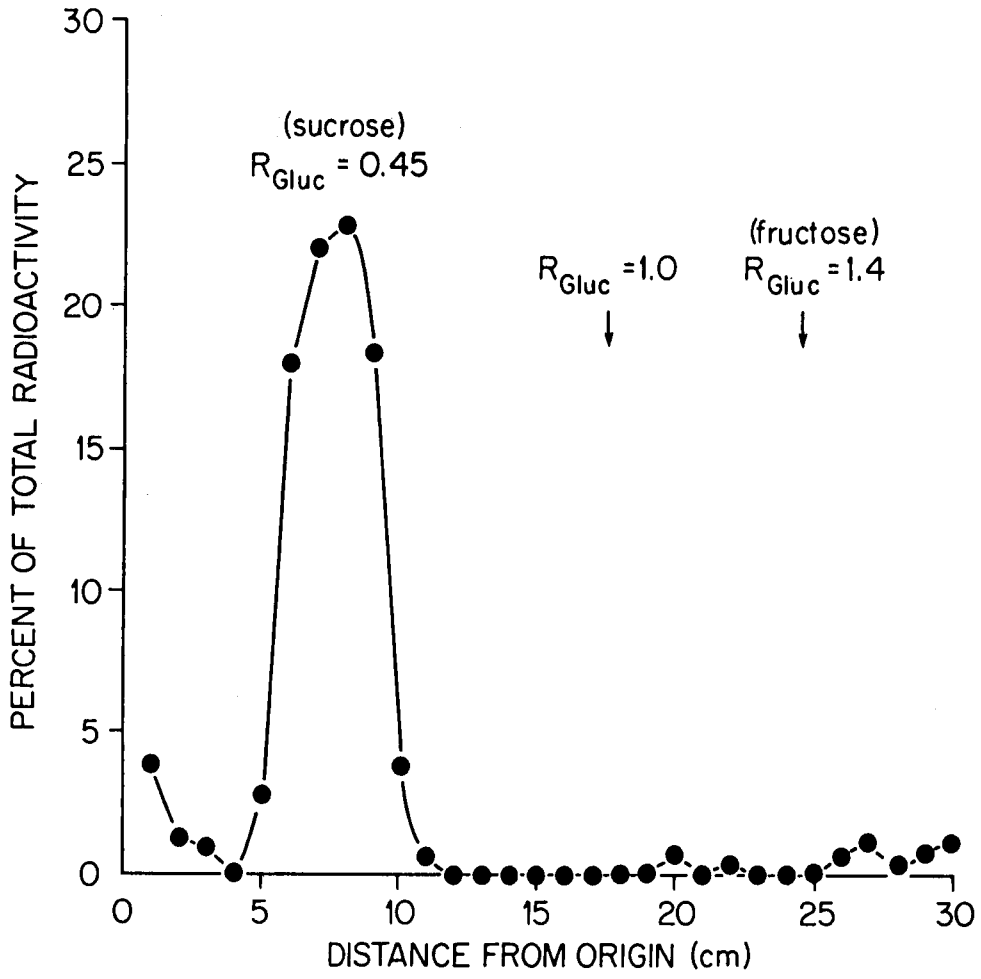


Fig. IV-3. Chromatography of cell extracts from [^{14}C]sucrose-loaded cells.

HeLa cells were incubated at 37°C for 15 hr in growth medium containing 1 μCi/ml purified [^{14}C]sucrose. Cell samples were then harvested and washed as described in Chapter II. Cells were lysed by sonication in a small amount of H_2O. The samples were then centrifuged at 2000 × g to eliminate some cell debris. The supernatant, which contained 100% of the cell-associated radioactivity, was then streaked on Whatman 3 MM paper strips and run in the chromatography system as described in Chapter II. ([^{14}C]sucrose purification and chromatography of cell extracts). The normal migration distances for glucose and fructose are indicated by the arrows.

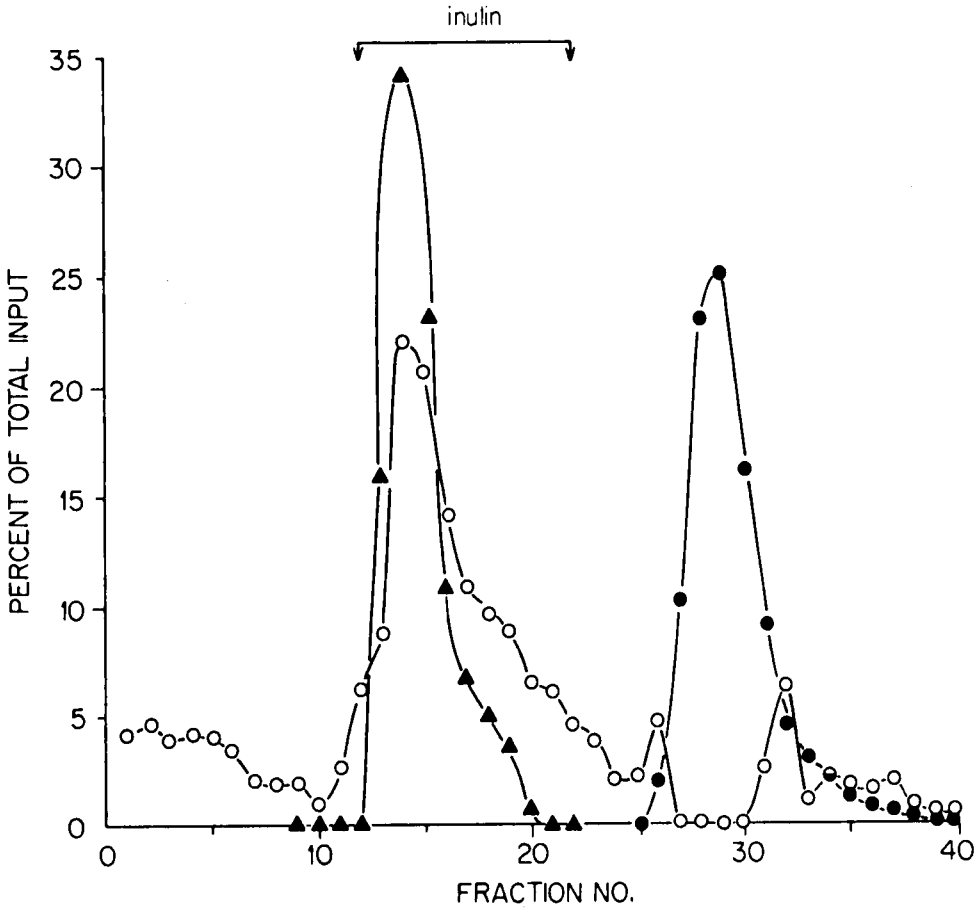


Fig. IV-4. Chromatography of a lysate of inulin-loaded HeLa cells. HeLa cells were labeled with [^{3}H]inulin for 15 hr at 37°C. Cell samples were then harvested and washed as described in Chapter II. The cells were lysed osmotically in 0.01 M sodium phosphate buffer, applied to a G-25 fine column, and fractions were collected as described in Chapter II (0-0). The void volume was determined with blue dextran (▲-▲) and the total column volume with $^{86}R6^{+}$ (●-●). The normal elution profile for inulin (mol. wt. = 5000) is indicated by the arrows.

particulates cannot be ruled out. Finally, previous chromatographic results showed that ouabain, the marker for adsorptive macropinocytosis, is not metabolized by HeLa suspension cells (Will et al., 1977).

CHAPTER V

EFFLUX OF PINOCYTIC MARKERS

Most reports of pinocytic marker efflux from cells have been in conjunction with studies of rates of intralysosomal digestion (Ehrenreich and Cohn, 1967, 1968, 1969; Steinman et al., 1974). Some evidence exists that nondigestible tracers for pinocytosis are eventually lost from the cells (Wattiaux et al., 1963; Bradford et al., 1969; Hers et al., 1973). However, other investigators have found that these materials remain with cells "indefinitely" (Ehrenreich and Cohn, 1967; Vernon-Roberts, 1972; Brunk, 1973; Carr, 1973; Novikoff, 1973; Davies et al., 1973b). In this chapter, I demonstrate that three nondigestible pinocytic tracers are lost at measurable rates from HeLa suspension cells. The efflux of internalized ouabain, the marker for adsorptive macropinocytosis, is first order, while that of the micropinocytic tracers, sucrose and inulin, has two components, a small (∿20% of the total efflux) rapid efflux and a slow efflux. Possible mechanisms for the two efflux components are also discussed.

Kinetics of Marker Efflux

The average half-time for the fast component (F) of sucrose efflux is 40 min and for the slow component (S) is 54 hr (Table V-1). Component F makes up an average of 18% of the total efflux Fig. V-1 shows that the kinetics of sucrose efflux are identical for cells labeled 2 hr or 15 hr. This indicates that, if there is a specific sequence of events

TABLE V-1

AVERAGE HALF-TIMES FOR PINOCYTIC MARKER EFFLUX[a]

Marker	T 1/2, min Component F	T 1/2, hr Component S	Percent of Total Efflux Represented by Component F
Sucrose	40 ± 6 (n = 6)	54 ± 4 (n = 6)	18 ± 7 (n = 6)
Inulin	60	125	6
Ouabain	—	60 ± 8 (n = 2)	—

[a]Sucrose, inulin, and ouabain effluxes were measured as described in Fig. V-1, V-2, and V-3, respectively. Results are expressed as the average half-times measured for n experiments ± S.E.M.

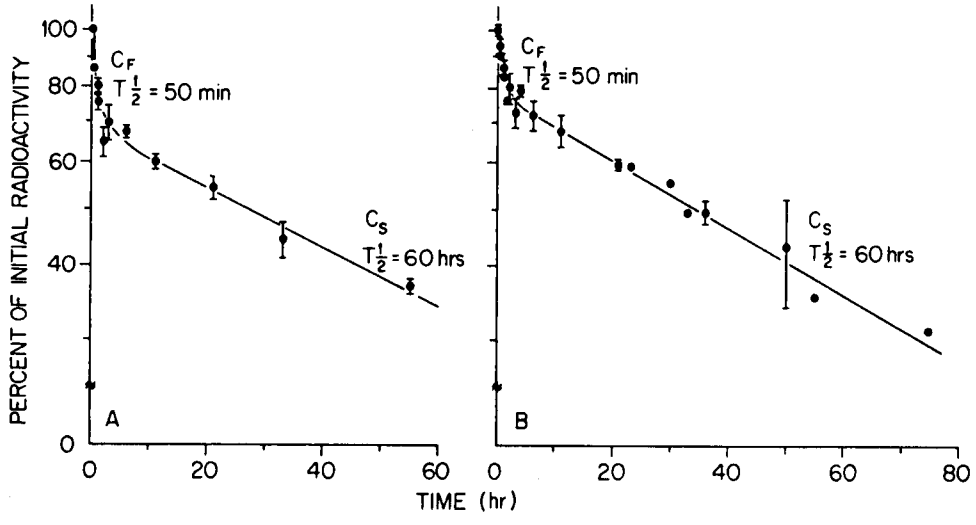


Fig. V-1. Sucrose efflux from HeLa suspension cells after different influx times.

C_F = Component F; C_S = Component S. A. Cells were labeled at 37°C in growth medium with 1 μCi/ml [^{14}C]sucrose for 2 hr. The cell suspension was centrifuged and resuspended in fresh nonradioactive growth medium at "0 time." Triplicate samples were harvested at the indicated times and assayed for cell-associated radioactivity as described in Chapter II. Results are expressed as percent of initial ("0 time") cell-associated radioactivity and error bars are ± one S.D. in all Chapter V figures. B. Cells were labeled and treated as for Fig. V-1A except that the label time was 15 hr and the [^{14}C]sucrose concentration during labeling was 0.5 μCi/ml.

that micropinosomes must undergo before efflux can take place, it must require less than 2 hr.

The half-times for the fast and slow components of inulin efflux are 60 min and 125 hr respectively (Fig. V-2; Table V-1). The fast component half-time is within the range of values obtained for component F of sucrose efflux. However, the fast component of inulin efflux makes up only about 6% of the total efflux, while component F of sucrose efflux comprises 18% of the total. The 125 hr half-time for component S of inulin efflux is twofold greater than the largest value obtained in any experiment for sucrose efflux component S. Since inulin and sucrose seem to be taken up by the same mechanism (Chapter III), there is no reason to think that their effluxes would be by different mechanisms. More inulin efflux experiments should be done to determine whether the observed differences between inulin and sucrose efflux are statistically significant.

Fig. V-3 shows that internalized ouabain efflux is first order, and that the half-time of efflux is the same as that of the slow component of sucrose efflux. This indicates that ouabain and sucrose efflux may be by the same mechanism, although there are differences in their mechanisms of uptake (Chapter III). Presumably in HeLa cells all classes of endocytic vesicles fuse with lysosomes inside the cell and eventually are found in the same intracellular pool from which efflux takes place.

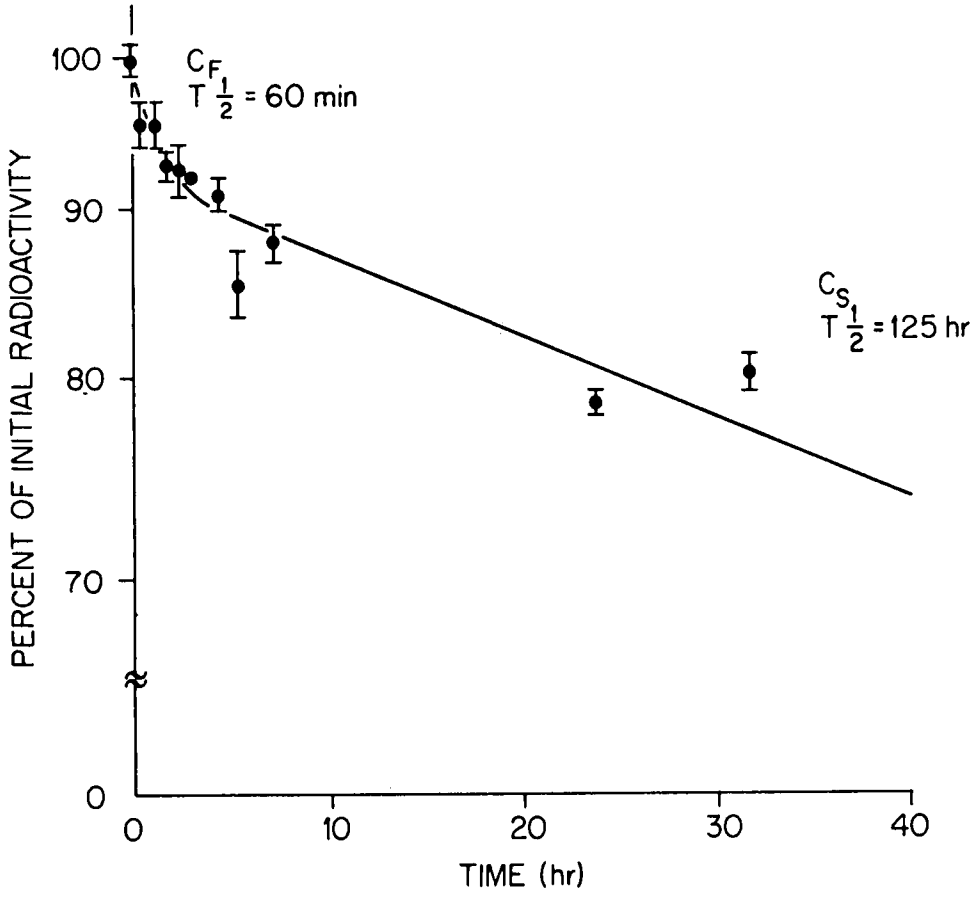


Fig. V-2. Efflux of inulin from HeLa suspension cells.

A cell suspension was labeled at 37°C for 15 hr in growth medium with 0.5 μCi/ml [^{14}C]inulin. The labeled cell suspension was then treated as described in Fig. V-1. C_F = Component F; C_S = Component S.

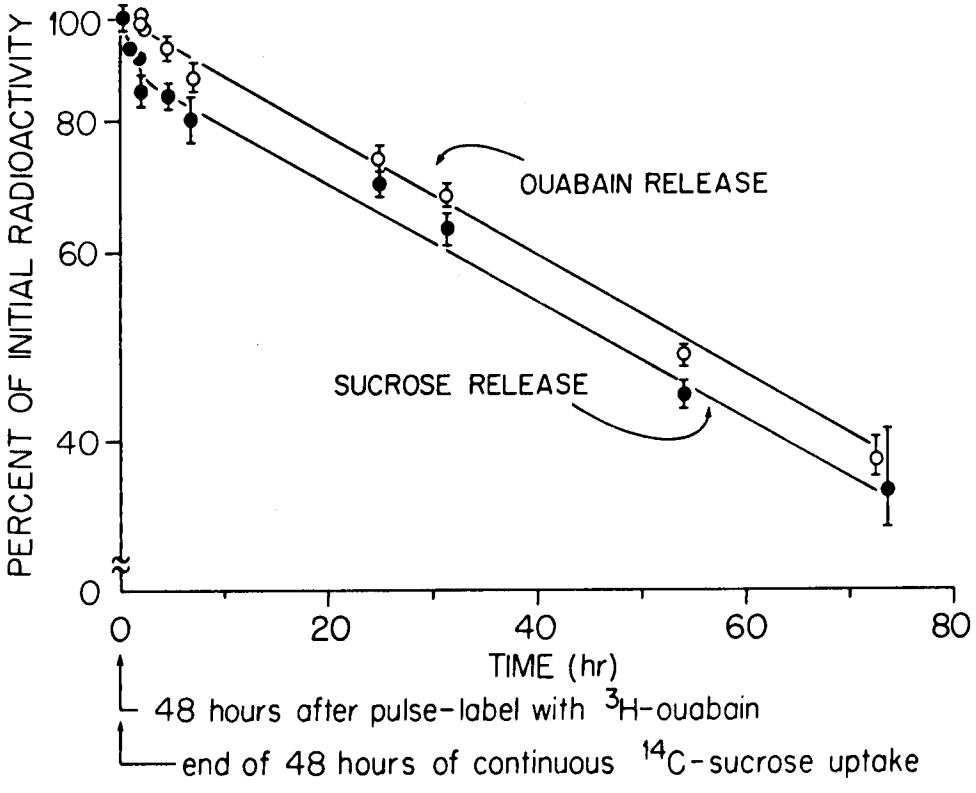


Fig. V-3. A comparison of sucrose and internalized ouabain release from HeLa suspension cells.

Cells were pulse labeled for 15-20 min at 2×10^{-7} M [^{3}H]ouabain in growth medium with 0.55 mM KCl instead of 5.5 mM KCL (see Fig. IV-1). The cell suspension was then centrifuged and resuspended in non-radioactive growth medium. [^{14}C]sucrose was added at a concentration of 1 μCi/ml. The cells were incubated under normal growth conditions for 48 hr, centrifuged, and resuspended again in fresh growth medium. Samples were harvested and assayed for cell-associated radioactivity as described in Chapter II at the indicated times after removal of sucrose. Sucrose ●-●; Ouabain 0-0.

Alternative Explanations for the Fast Component (F)

Three possible explanations for efflux component F are: (1) dissociation of marker bound to the cell membrane; (2) diffusion of free intracellular marker out of the cells possibly induced by centrifugation and resuspension of the cells by pipetting; and (3) refusion with the plasma membrane of micropinosomes that are still closely associated with the membrane.

The first explanation, dissociation of bound marker from the cell membrane, is not plausible since it was shown in Chapter III that neither sucrose nor inulin binds to HeLa suspension cells. Component F cannot be diffusion of soluble intracellular tracer from the cells caused by removal of extracellular tracer. The sucrose influx experiments described in Chapter III show that sucrose does not diffuse across the cell membrane with a half-time of 40 min.

Diffusion from a soluble intracellular pool might be induced by centrifugation and resuspension of the cells by pipetting. The experiment described in Fig. V-4 was designed to test this hypothesis. A cell suspension was labeled for 15 hr in [^{14}C]sucrose, centrifuged, and resuspended in fresh growth medium for 3 hr (efflux A). This provided sufficient time for the completion of the fast efflux component. The cells were then centrifuged and suspended again in fresh growth medium (efflux B). No fast efflux component was observed during efflux B. Therefore, centrifugation and resuspension of the cells was not the sole inducer of efflux component F.

A third possible explanation for component F is illustrated in Fig. V-5. Micropinosomes which are still partially attached to the

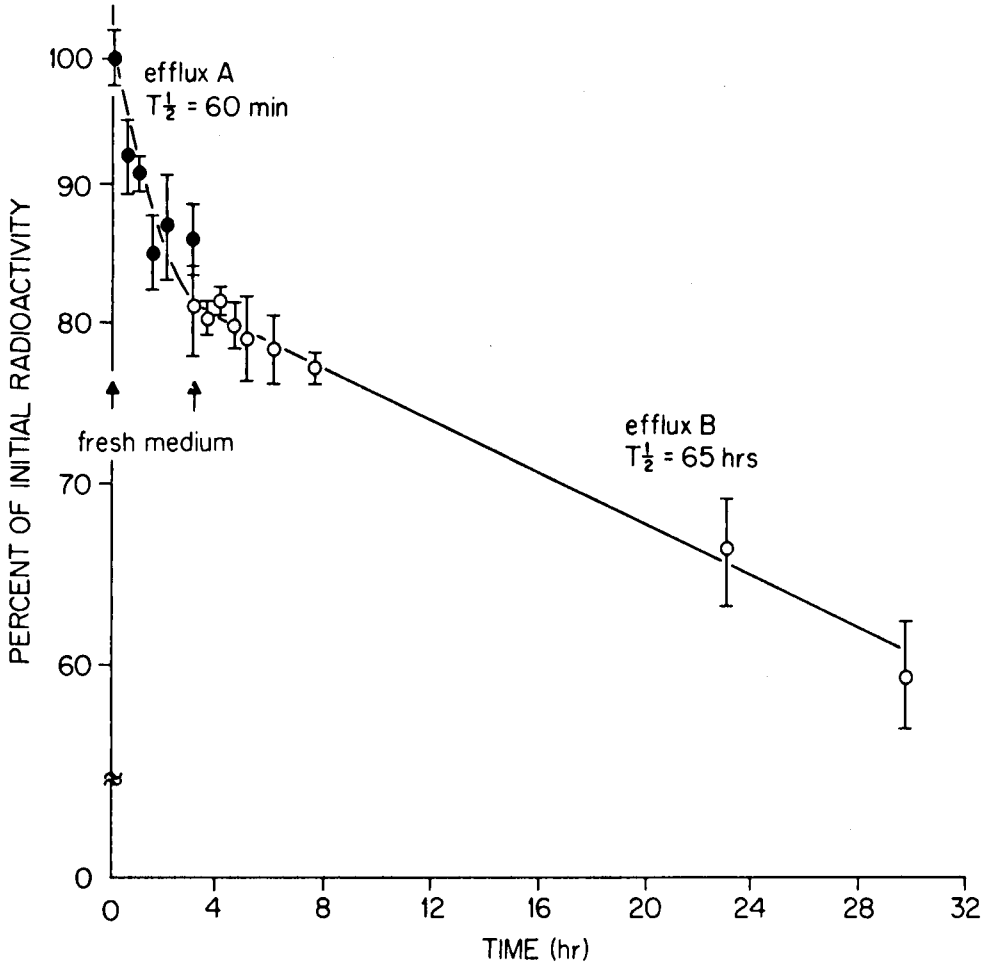


Fig. V-4. Centrifugation and resuspension of [^{14}C]sucrose-loaded HeLa cells does not induce Component F of sucrose efflux.

A cell suspension was incubated at 37°C in growth medium with 0.5 μCi/ml [^{14}C]sucrose for 15 hr. The suspension was then centrifuged and resuspended in fresh nonradioactive growth medium. Triplicate samples were harvested at the indicated times and assayed for cell-associated radioactivity as described in Chapter II. After 3 hr, the cell suspension was again centrifuged and resuspended in fresh nonradioactive growth medium (indicated by the arrow). Triplicate samples were again harvested and assayed for radioactivity at the indicated times. The first 3 hr efflux period is called efflux A and is indicated by ●-●. The second efflux period (3-23 hr) is labeled efflux B and is indicated by 0-0.

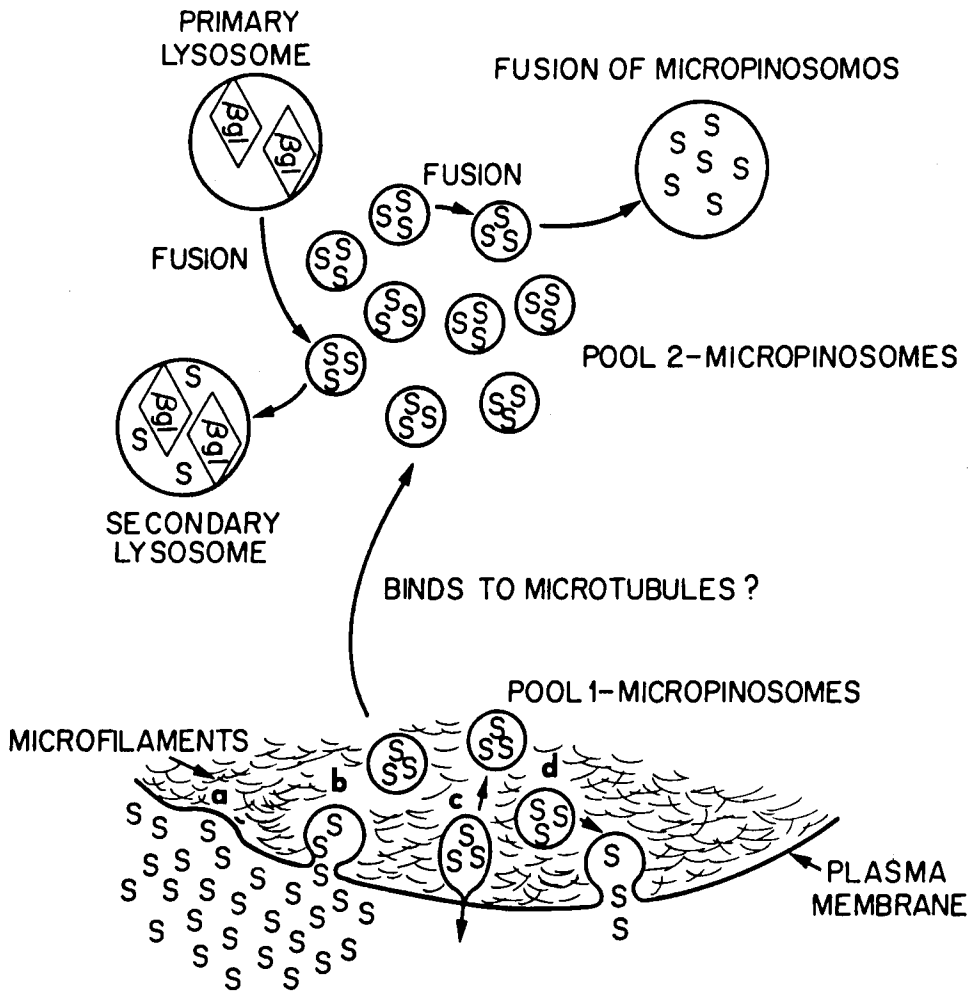


Fig. V-5. Micropinocytosis of sucrose in HeLa cells and a model for Component F of sucrose efflux.

S = sucrose; Bgl = lysosomal marker enzyme, N-acetyl-β-glucosaminidase. The drawing starts with a forming micropinosome (a). Sucrose is trapped inside the micropinosome, which then breaks free of the membrane (b) and enters pool 1 micropinosomes. Some micropinosomes which are still attached to the membrane (c) or are trapped in the microfilament system (d) may refuse with the plasma membrane instead of passing into the cell interior to pool 2 (see text). Access to pool 2 may involve binding of micropinosomes to microtubules and subsequent transport by these structures to the cell interior (pool 2). After the micropinosomes reach pool 2, they may fuse with other micropinosomes and/or primary lysosomes to form secondary lysosomes.

plasma membrane or in close proximity to it are represented by pool 1. These vesicles might be able to refuse with the plasma membrane. Centrifugation and resuspension by pipetting might induce this refusion and subsequent expulsion of micropinosome contents into the medium. The absence of component F during efflux B (Fig. V-4) suggests that 3 hr is sufficient to "chase" micropinosomes from hypothetical pool 1 to pool 2. The lack of a fast component of internalized ouabain dissociation is also consistent with this model. Since ouabain binds to HeLa plasma membranes, a "chase" period was required for clearance of ouabain from the plasma membrane, so that efflux measurements were uncomplicated by ouabain dissociation from the membrane. After the 48 hr "chase" period used in the experiment shown in Fig. V-3, p. 53, there would be no pool 1 micropinosomes. Pool 1 must saturate fairly rapidly, since the percentage of total intracellular marker molecules associated with component F after 2 hr of sucrose uptake was not much larger than after 15 hr uptake (Fig. V-1, p. 50). These results are consistent with those of Steinman and coworkers (1976) who found that the intracellular pinocytic vesicle space saturated in L cells after 5 min of HRP uptake.

There are several possible mechanisms for movement of vesicles from hypothetical pool 1 to pool 2. First, Allison (1974) has suggested that microfilaments normally serve as a barrier to fusion of intracellular vesicles with the plasma membrane, since cytochalasin B stimulates exocytosis in some systems. The converse might happen during micropinocytosis; the fraction of micropinosomes which make

up the fast component might be vesicles which are held in close proximity to the plasma membrane by the microfilament system. An alternative, which is not necessarily exclusive of the first mechanism, is that the initial step in transport from pool 1 to pool 2 might be binding of the vesicles to microtubules. Vesicles which have not yet bound to microtubules might be free to refuse with the plasma membrane expelling their contents back into the medium. The transport to the cell interior by saltatory motion involving microtubules is well documented (Freed and Lebowitz, 1970; Bhisey and Freed, 1971).

Possible Explanations for the Slow Efflux Component

The mechanism of the slow efflux component of inulin and sucrose and the first order efflux of ouabain is unknown. The markers might remain vesicle-bound indefinitely inside the cell and escape only by random refusion with the plasma membrane. Very little is known about this process, called exocytosis, in relation to excretion of non-digestible materials. Exocytosis is the known mechanism by which hormone secretion occurs (Palade, 1959; Jamison and Palade, 1967; Amsterdam et al., 1969; Bennet, 1969; Farquhar, 1969; Pelletier et al., 1971; Mazur et al., 1972; Orci et al., 1977) and is probably also the mechanism of neurotransmitter release (Norman, 1976).

A second possible mechanism of the slow efflux is release of the marker from the vesicle intracellularly and its subsequent passive diffusion from the cytoplasm to the medium. Data presented in Chapter IV (Fig. IV-1, p. 39), indicating that there is very little

internalized ouabain found in soluble form, makes this explanation seem unlikely.

CHAPTER VI

MICROPINOCYTOSIS DURING THE CELL CYCLE

In some cell types, there is scanning electron microscopic evidence that a specific surface morphology corresponds to each stage of the cell cycle (Porter et al., 1973; Everhart and Rubin, 1974; Enlander et al., 1975; and Collard and Temmink, 1976). Since micropinocytosis is a surface activity and may be dependent on cell surface conformation, it seemed likely that this activity might undergo morphologically related rate changes during the cell cycle. Quintartx et al. (1976) have shown that micropinocytosis measured by HRP uptake in cultured hepatoma cells increased fivefold to tenfold during G_1. In contrast, my results with HeLa S3 suspension cells showed no variation in micropinocytic rate during the cell cycle.

Fig. VI-1A shows a representative cell synchrony experiment in which > 90% division occurred. DNA synthesis (S) started at 3-4 hr and cell division at about 8 hr after release from the second thymidine block of DNA synthesis (see Chapter II).

Fig. VI-1B shows that cell volume increased throughout the cell cycle and peaked just before the beginning of cell division (G_2). After the completion of division, the G_1 cell volume was approximately half of the G_2 volume. Micropinocytic rate followed a pattern similar to cell volume except that the peak uptake was in mitosis (M) instead of in G_2.

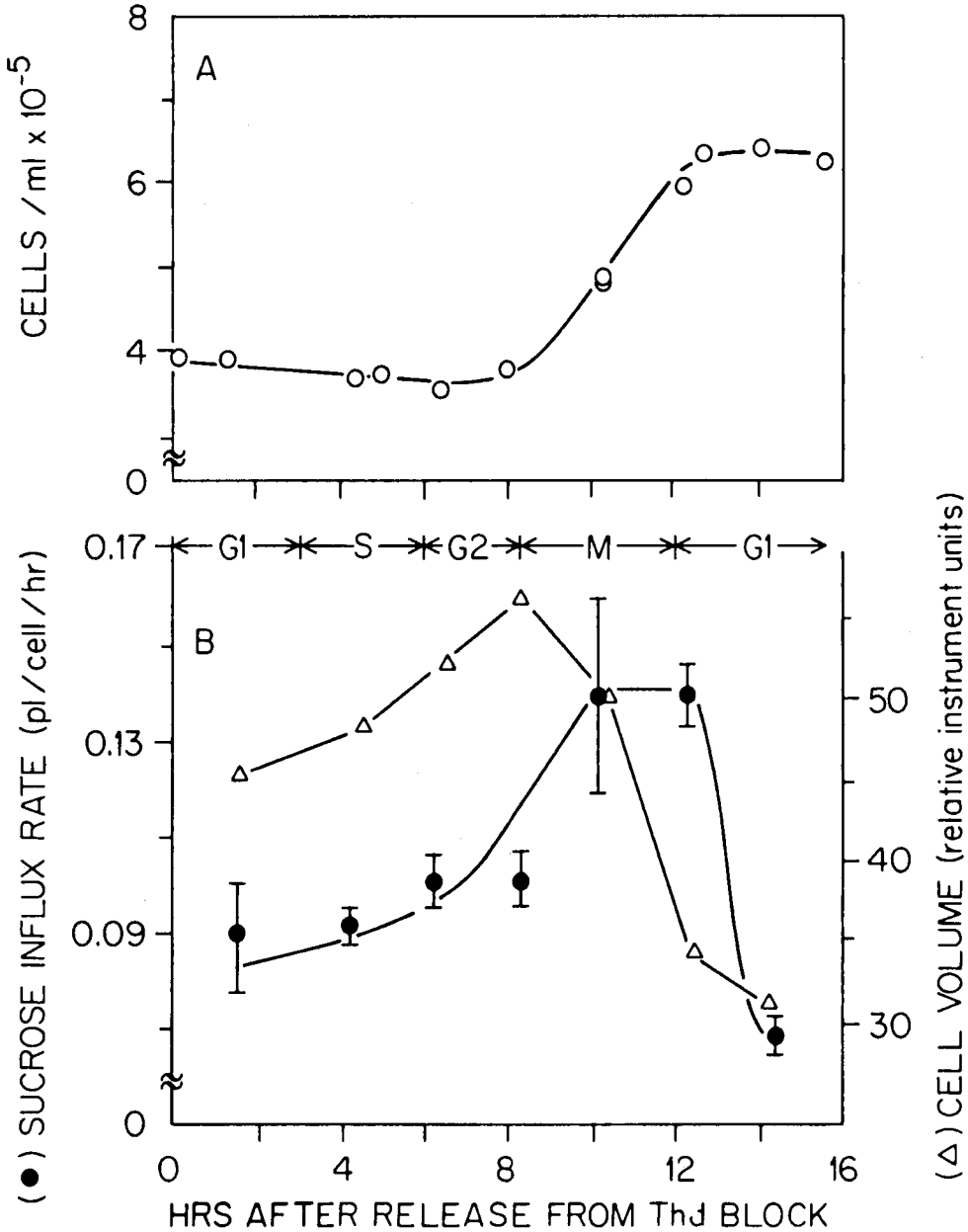


Fig. VI-1. HeLa suspension cell volume, number, and micropinocytic rate during the cell cycle.

Cells were synchronized by the double thymidine block method described in Chapter II. The S period was determined by assay for [^{3}H]thymidine incorporation into TCA insoluble radioactivity at various times after release from the second thymidine block. A. Cell number was determined with a Coulter counter. B. Cell volume was determined with a Coulter Channelyzer, and results are expressed as the mean instrument window number (relative instrument units). Micropinocytosis was assayed by uptake Method I (Chapter II) using [^{14}C]sucrose at 1 μCi/ml. The average of triplicate "0 time" samples was substracted from the average uptake after 60 min to determine micropinocytic rate. Rates are expressed as pℓ medium micropinocytosed/cell/hr (see Chapter II).

In the experiment described in Fig. VI-1B, micropinocytic rate was measured by uptake assay Method I (Chapter II). An average "0 time" uptake was subtracted from an average 60 min uptake to obtain the initial micropinocytic rate. The points in Fig. VI-1B were arbitrarily plotted as the starting time for each 60 min uptake assay.

The uncertainty related to the proper time to plot in Fig. VI-1B and complications caused by mechanical manipulation of the cells with uptake assay Method I were avoided by labeling the cells continuously with sucrose or inulin after removal of the second thymidine block (Fig. VI-2). Changes in micropinocytic rate would be detected in such an experiment by a deviation in the linear uptake curve. Fig. VI-2 shows, however, that no deviation in the linearity of sucrose or inulin uptake was observed for 16 hr after release from the second thymidine block of DNA synthesis.

I conclude from the above observations that: (1) micropinocytic rate is constant in HeLa suspension cells throughout the cell cycle, and (2) the peak of micropinocytosis in M observed in my early experiments was probably an artifact of the experimental design. It might be due to an enhanced diffusional uptake during mitosis stimulated by the centrifugation and/or pipetting of cells with assay Method I. Cells might be more sensitive to mechanical manipulation during cytokinesis when the dividing cells actually pull apart stretching the membrane and possibly making it transiently leaky.

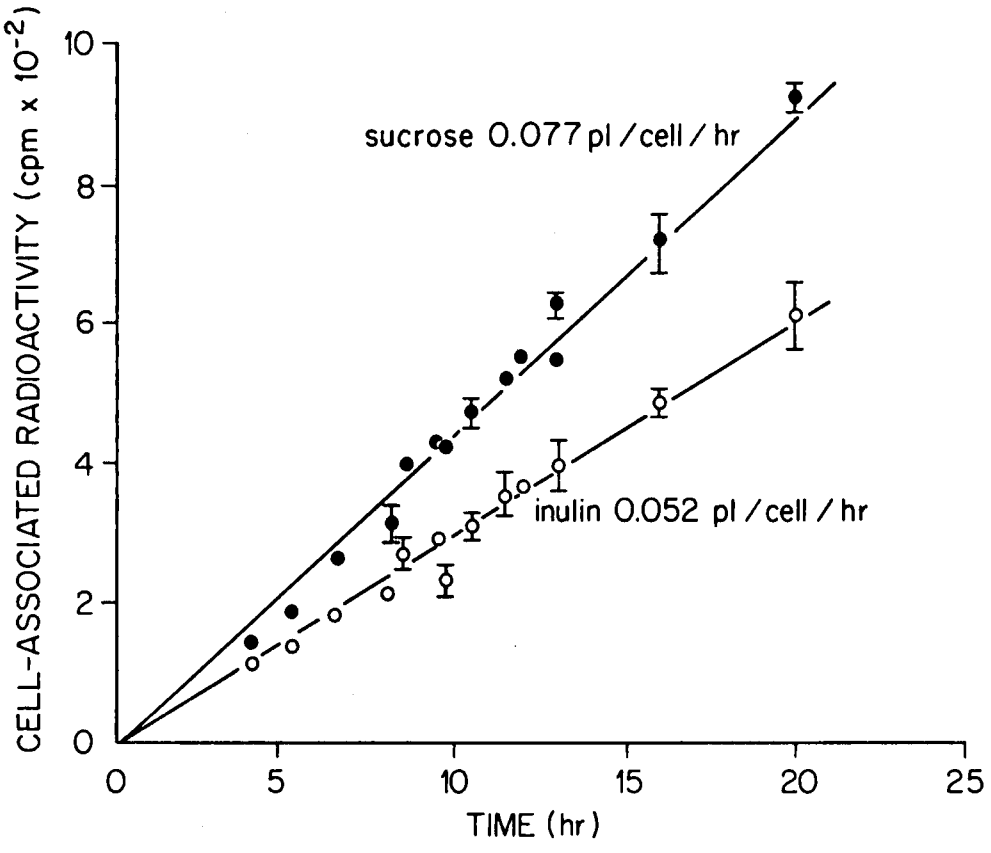


Fig. VI-2. Micropinocytosis of inulin and sucrose during the cell cycle.

[^{14}C]inulin or [^{14}C]sucrose at a concentration of 0.5 μCi/ml was added immediately after release from the second thymidine block. Triplicate samples were harvested as described in Chapter II at the indicated times. Results are expressed as cell-associated radioactivity/sample aliquot.

CHAPTER VII

PREPARATION OF SUSPENSION-CULTURED MAMMALIAN CELLS FOR SCANNING ELECTRON MICROSCOPY (SEM)

A suitable method for the preparation of HeLa suspension cells for SEM was necessary to determine experimentally whether HeLa cell surface structure changes during the cell cycle and whether a particular cell cycle specific surface conformation correlates with a high rate of micropinocytosis. In Chapter VI, it was shown that there was no variation in micropinocytic rate during the cell cycle. Therefore, I did not extensively pursue the SEM related experiments.

In this chapter, I present (1) progress toward the development of an easy method for preparation of HeLa suspension cells for SEM; (2) some observations related to volume changes during SEM preparation, volume regulation after osmotic stress, and osmotic responsiveness after glutaraldehyde fixation; and (3) data which indicate that there is little variation in surface morphology of HeLa suspension cells during the cell cycle. It was desirable to fix cells while still in suspension to avoid possible attachment-related surface morphological changes. Several preparatory methods have been reported for SEM of suspension cells: (a) collection of the cells by filtration onto a "Flotronic" silver membrane filter (De Harven et al., 1973); (b) fixation and processing of the cell suspension in a chamber made up of two gold-coated nucleopore filters separated by a set of brass washers (Nemanic et al., 1975); (c) collection of fixed suspension cells on

coverslips coated with a monolayer of normal live cells (Lin et al., 1973; Collard and Temmink, 1975) or a substance, such as polylysine (Mazia et al., 1975; Sanders et al., 1975) or 3,3 ionine chloride (Molday et al., 1975), that is positively charged at neutral pH and will electrostatically attract the fixed cells. The method described in this chapter is a modification of the latter method employing polylysine-coated microslides.

SEM Preparation Method

Preparation of microslides. Microslides (1 cm^2) were cut from 75 × 25 mm microscope slides. It was desirable to use these microslides instead of the thin precut coverslips, since the former are not fragile, do not slide over each other, and fit well onto standard SEM specimen stubs. The microslides were cleaned by boiling once in 1% 7X Detergent (Linbro) and 3 times in glass distilled water with copious rinsing with distilled water between boilings. Acid treatment (1 M HCl for 1 wk) of the microslides after washing decreased the efficiency of coating with polylysine and the subsequent attachment of fixed cells. Alcoholic KOH or NaOH (1 M) pretreatment did not effect coating and attachment. Therefore, microslides were not further treated after washing and before coating with polylysine. The microslides were soaked for a few minutes in 0.5% aqueous polylysine (D, L, or DL isomers), removed, and rinsed in distilled water. Extensive rinsing, or rinsing in running water, was avoided since this procedure removed too much polylysine. Failure to rinse at all, however, caused

an uneven distribution of polylysine and subsequent peeling of thick layers of the polycation during later steps in the procedure. The coated and rinsed microslides were then placed in an aluminum foil tray (about 0.5 cm deep), which was put into a 60 mm petri dish.

Fixation of suspension HeLa cells in glutaraldehyde. A suspension of HeLa cells in growth medium was fixed by the dropwise addition of an equal volume of 2% glutaraldehyde in serum-free S-EMEM. Since temperature changes alter surface structure in unfixed cells (Lin et al., 1973), all reagents were at 37°C at the time of addition of the fixative. The cell suspension (usually 10 ml at about 1.5×10^5 cells/ml) was poured into the 60 mm petri dish containing the polylysine-coated microslides, and the cells settled while fixation continued for 30-60 min. This procedure was carried out at 25°C, since a control experiment showed that settling, adherence, and cell morphology was the same at 25°C and 37°C.

Osmium tetroxide post-fixation was not used routinely since there was no difference observed between post-fixed cells and cells which were fixed only in glutaraldehyde.

Dehydration and critical point drying. The glutaraldehyde fixed cell suspension was made 10% in ethanol by the removal of a small amount of glutaraldehyde-medium and the addition of the same volume of 100% ethanol, added dropwise. This process was repeated with 10% step intervals until a concentration of 30% ethanol was reached. The cells were left at each concentration for about 5 min. The

glutaraldehyde-medium-ethanol solution was then aspirated from the petri dish (but not from the aluminum foil tray) and replaced with 40% ethanol. This process was repeated with increasing concentrations of ethanol in steps of 10% until 100% was reached. Three changes of 100% ethanol were made. No precipitation of protein by the ethanol occurred during this procedure. Also no morphological difference was noted when dehydration was carried out with acetone instead of ethanol. The microslides in the holder were then critical point dried as described in Chapter II.

Evaluation of SEM preparation method. There are several convenient features to the method described above. First, > 90% of the cells that adhere to the polylysine coating on the microslides remain attached throughout the preparation procedure. Second, the procedure produces a minimum amount of distortion or loss of cells which usually results from the transfer of the microslides with forceps through the fixation and dehydration solutions. Third, aspiration as a method of changing ethanol concentrations during dehydration and the use of the aluminum foil holder for the microslides eliminates surface tension forces which occur when microslides are transferred in a conventional Chen coverslip holder (Cat. No. 8542-F66, Arthur H. Thomas Co.), where microslides are held vertically. Finally, the procedure, except for critical point drying, is not time consuming. One outstanding problem, namely cell shrinkage during the preparation, will be discussed in the section that follows.

Volume changes during SEM preparation. It has previously been suggested that microvilli are "storage organs" for extra membrane, which accumulates as the cell enlarges during the cell cycle and which "unfolds" during cytokinesis (Lamb and MacKinnon, 1971; Knutton et al., 1975; Knutton et al., 1976). The following experiment was designed to test this hypothesis. Control cells were fixed and prepared for SEM as described above. Experimental cells were swelled by a 2 min incubation in hypotonic growth medium (equal parts growth medium and distilled water). These conditions produced the maximum possible cell volume as determined by the Coulter Channelyzer. The cells were then processed for SEM as described above. The fixative used was 2% glutaraldehyde in 50% serum-free S-EMEM. If the membrane-storage organ hypothesis of microvillus function is correct, the maximally swelled cells should be devoid of microvilli, and the resulting smooth-surfaced cells should have the maximum diameter and volume possible without cell lysis. Instead, we found that the maximally swelled cells had microvilli and the same diameter as the controls (Figs. VII-1, VII-2). The average diameter from 50 such measurements is given in Table VII-1. The cell volume calculated from this average diameter was only 26% and 20% of the volume determined for controls and experimentals respectively with the Coulter Channelyzer. The value of 2000 μ^3 as determined by the latter method is in agreement with the value (1800 μ^3) obtained by direct cytocrit methods (Proctor, 1974). In an experiment similar to the one described above, Knutton et al. (1976) found that swelled Lettree cells had no microvilli.

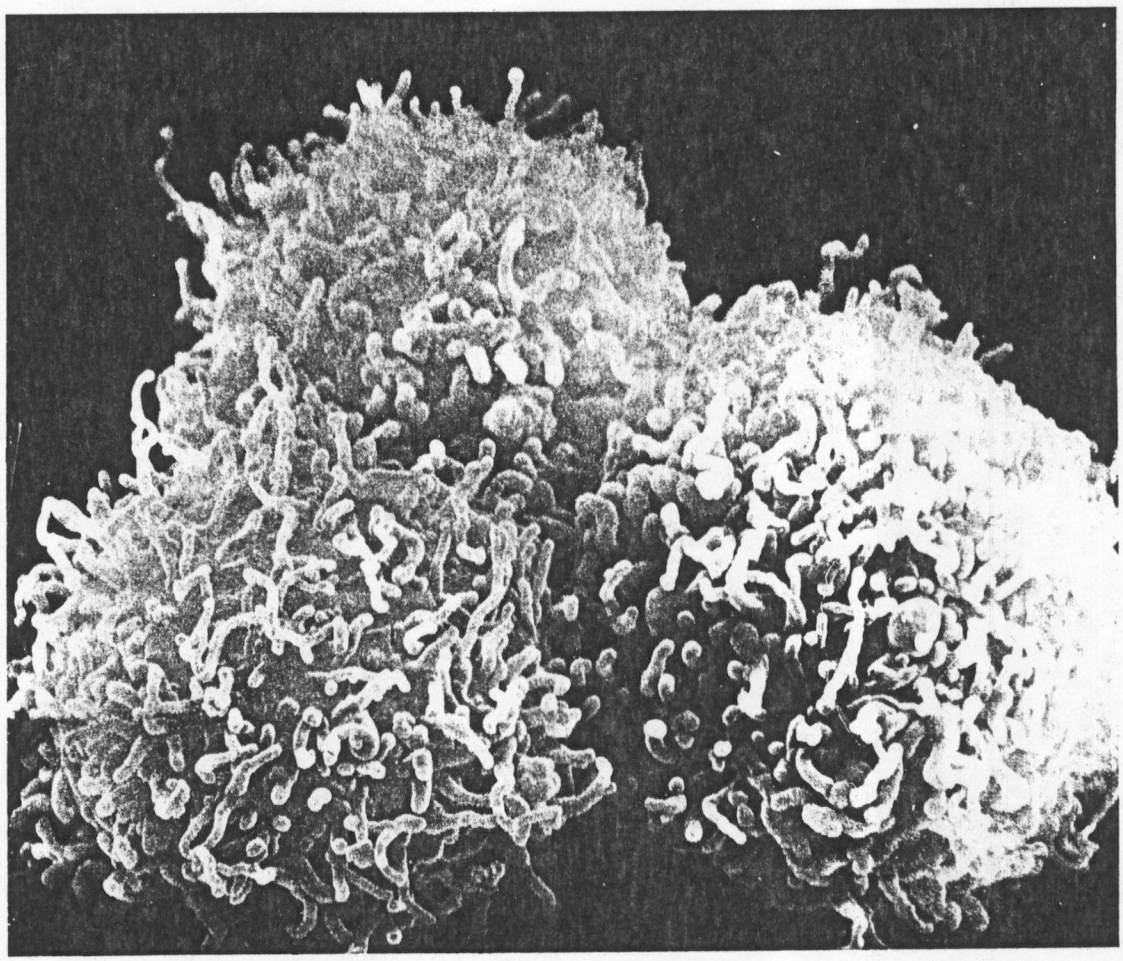


Fig. VII-1. Scanning electron micrograph of control HeLa suspension cells.

Cells prepared for SEM as described in the text.

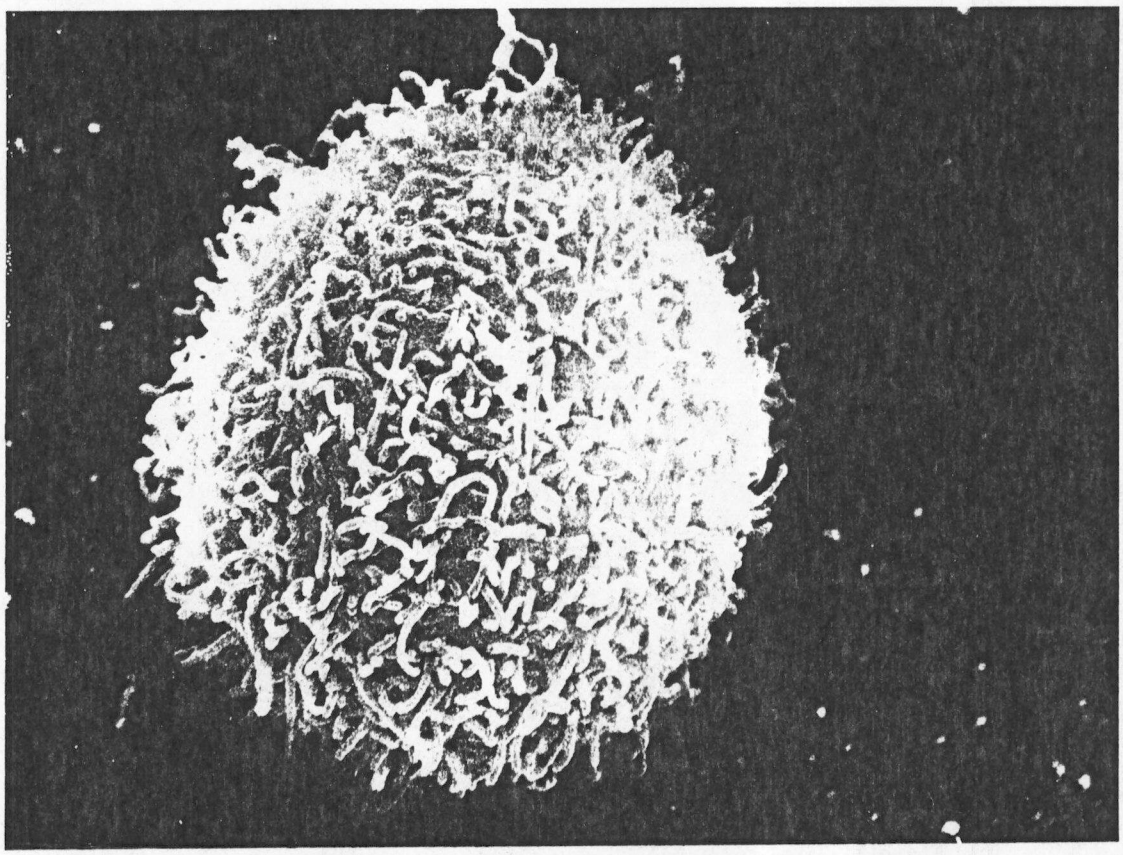


Fig. VII-2. Scanning electron micrograph of experimentally swelled HeLa suspension cells.

Cells were incubated at 25°C in a 1:1 mixture of growth medium and distilled water prior to fixation as described in the text except with 2% glutaraldehyde in 50% S-EMEM as the fixative. The remainder of the preparative procedure was identical to that used for the controls in Fig. VII-1.

TABLE VII-1

A COMPARISON OF VOLUMES MEASURED WITH THE COULTER CHANNELYZER AND VOLUMES CALCULATED FROM DIAMETERS MEASURED WITH THE SEM[a]

Sample	Diameter, μ		Volume, μm^3	
	SEM	Channelyzer	SEM	Channelyzer
HeLa Cells				
Control	10.0 ± 0.2	15.3	520	1,980
Swelled	10.8 ± 0.9	18.0	660	3,280
Paper Mulberry Spores	12.3 ± 0.2	11.4[b]	970	770[c]
Lycopodium Spores	30.6 ± 0.2	27[b]	14,960	10,280[c]

[a]HeLa control cells and experimentally swelled cells were prepared for SEM as described in the text. Diameter measurements on SEM samples were of the cell body excluding the microvilli. The average of 50 such measurements is given ± S.E.M. Spores were prepared for SEM by affixing them to double-faced tape attached to a specimen stub. Diameter measurements were made and results expressed as described for HeLa cells. HeLa cell volume determinations with the Coulter Channelyzer were made using Paper Mulberry spores (11.4 μ diameter) for calibration. HeLa cell diameter was then calculated from the volume measurements.

[b]Diameter given by supplier.

[c]Volume calculated from diameter given by supplier.

However, in agreement with my results, the cell volume calculated from their control diameter measured in the SEM was only 30%-40% of the volume they determined with the Coulter counter. These results suggested two alternatives: (1) volume measurements made in the SEM are inaccurate because the limits of the cell surface are obscured by the presence of microvilli, and the diameter error is enhanced by d^3 in the volume calculation; or (2) some step in the preparation of cells for SEM causes cell shrinkage.

To test the first alternative, the diameter of Paper Mulberry and Lycopodium spores, which require no fixation and dehydration, were measured using the SEM. Table VII-1 shows that these diameters are within 10% of the diameter specified by the supplier. It is also worth noting that these diameter measurements were somewhat larger than their specified diameter, while HeLa cell diameter was much smaller than the value estimated from the Coulter Channelyzer measurements.

What step in the SEM preparation procedure might cause shrinkage of the HeLa cells? Fig. VII-3 shows that the fixation conditions used did not cause the shrinkage, since the salt concentration during fixation was isosmotic. Brunk and his coworkers (1975) have demonstrated that glutaraldehyde contributes nothing to the effective osmotic pressure of the fixative solution. They harvested cells grown in a 300 milliosmolar (mosm) environment and fixed them in 2% glutaraldehyde (200 mosm) in 0.1 M cacodylate buffer (also ∿200 mosm; total osmolarity 400 mosm). Despite the hyperosmolarity, the cells swelled rather than

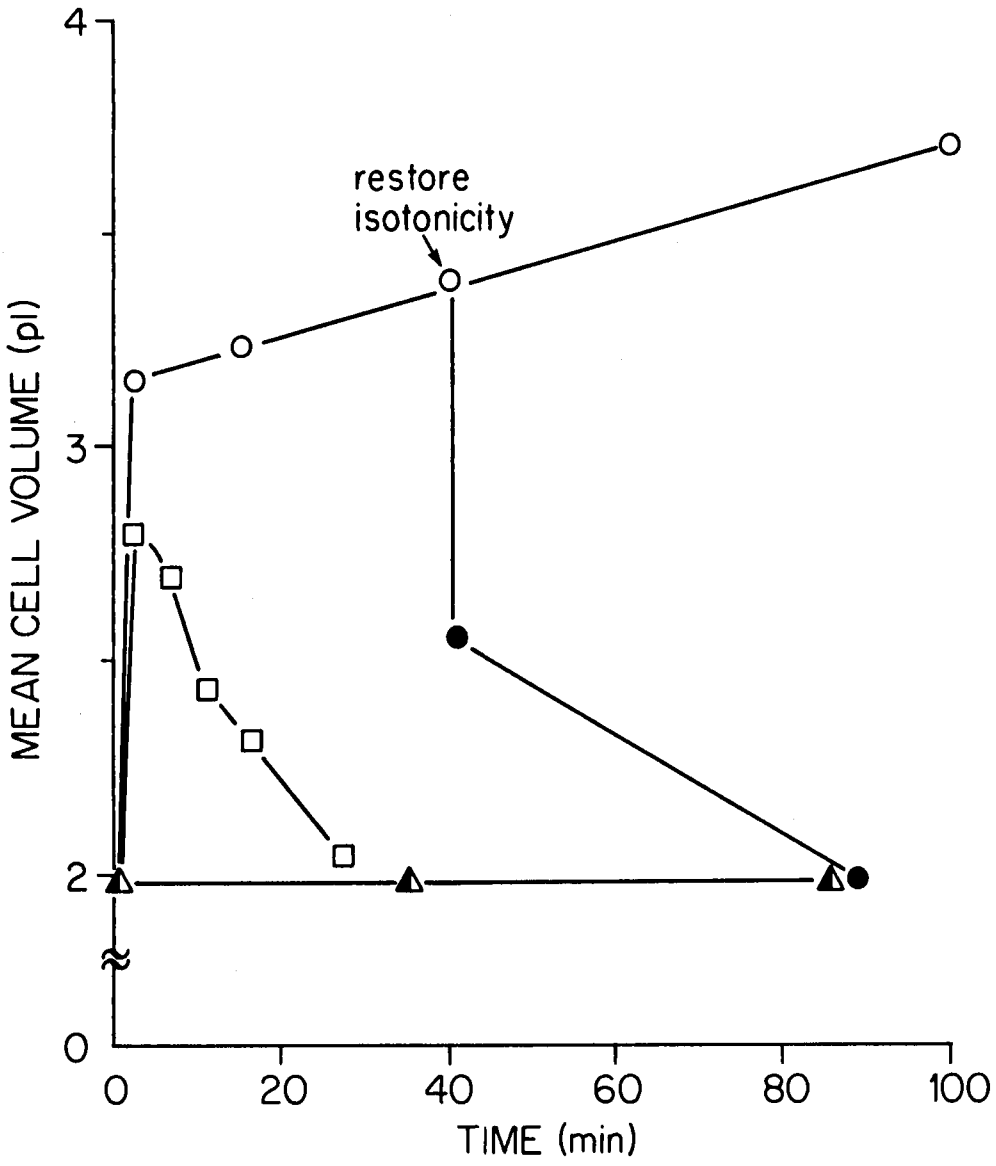


Fig. VII-3. Osmotic responsiveness of HeLa suspension cells after glutaraldehyde fixation.

Cell volumes were measured on the Coulter Channelyzer calibrated with Paper Mulberry spores (11.4 μ diameter). Unfixed controls (▲-▲) were incubated in S-EMEM at 25°C throughout the experiment. Fixed controls (Δ-Δ) were treated as described in the text and remained in fixative at 25°C for the duration of the experiment. Experimental cell samples were either incubated in a 1:1 mixture of S-EMEM and distilled water at 25°C throughout the experiment (□-□) or treated as described in Fig. VII-2 (0-0). A portion of the latter sample was restored to ∿320 m osmoles (isotonic for HeLa cells) at the indicated time (●-●).

shrank. Apparently the cells are freely premeable to glutaraldehyde which equilibrates rapidly; the effective osmotic pressure arises solely from the nonpermeating 200 mosm buffer.

In these studies on cell volume during fixation, it was observed that HeLa cells are osmotically responsive after glutaraldehyde fixation. As shown in Fig. VII-3, maximally swelled cells, which have been fixed in glutaraldehyde could be returned to their original volume by restoring an isosmotic salt concentration. Another interesting observation from Fig. VII-3 is that unfixed maximally swelled cells can actively restore their volume to normal without restoration to isosmotic conditions even at room temperature. This phenomenon has also been observed in other cell systems (Kregenow, 1972; Shank et al., 1972; Rosenberg et al., 1972).

Fig. VII-4 shows that probably no cell shrinkage occurred during ethanol dehydration. Slow addition of ethanol (up to 40%) to a fixed cell suspension as described above caused no volume changes detectable with the Coulter Channelyzer in control cells. In contrast to the controls, maximally swelled and fixed cells apparently did not maintain their swelled volume when ethanol was added dropwise. At first I thought that this observation might be an instrument artifact related to low medium conductivity, since conductivity is fundamental to the quantitative operation of the Coulter counter. However, results with Paper Mulberry spores under the same experimental conditions showed that medium conductivity did not become limiting until the salt concentration was reduced to 60-70 m<u>M</u>. The reason for the shrinkage

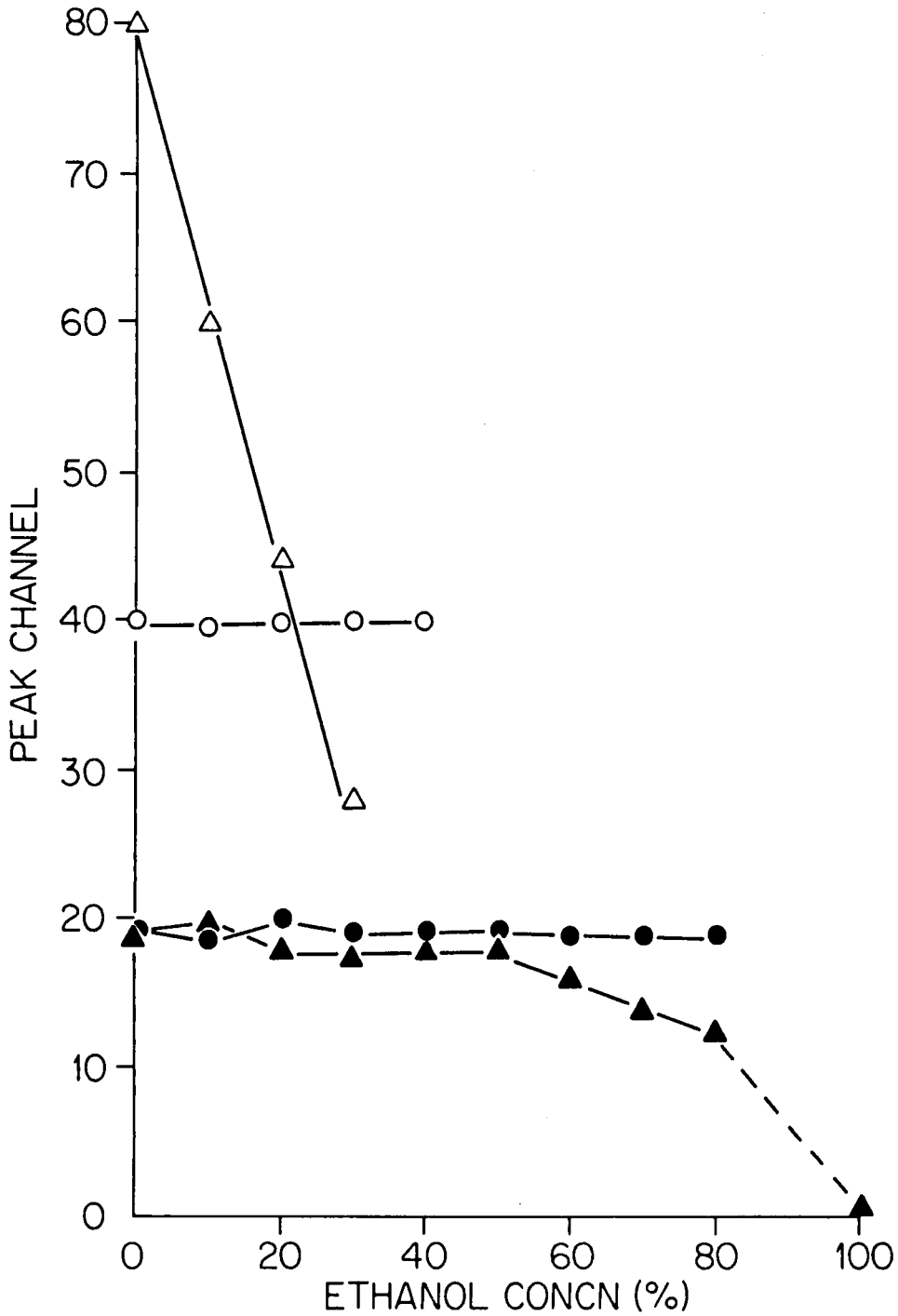


Fig. VII-4. The effect of ethanol dehydration on HeLa cell volume.

Cell volumes are expressed as the peak channel obtained with the Coulter Channelyzer. Some sample suspensions (control) were fixed as for Fig. VII-1 and then subjected to increasing ethanol concentration by the dehydration method described in the text. HeLa cells 0-0; Paper Mulberry spores ●-●. The remaining samples were treated in hypotonic medium as described for the experimentals in Fig. VII-2 prior to dehydration. HeLa cells Δ-Δ; Paper Mulberry spores ▲-▲.

of experimentally swelled and fixed cells and not the controls is unknown. However, it might be that ethanol permeabilizes the highly stretched membranes of swelled cells so much that the cells no longer cause as high a resistance in the Coulter counter as the control cells. The swelled cells would then appear artifactually smaller than the controls.

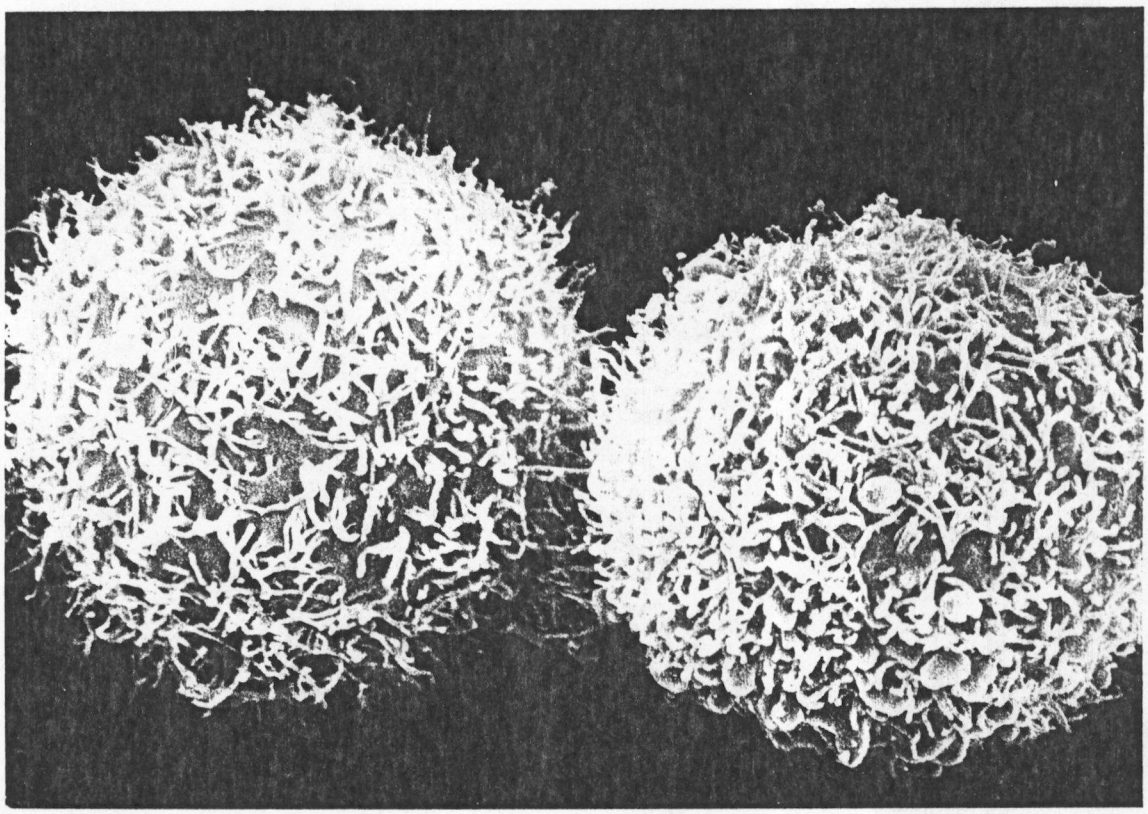
Finally these experiments show that the most likely step in SEM preparation for cell shrinkage to occur is the critical point drying. Shrinkage during this step has been observed in mouse liver nuclei (Gusnard and Kirschner, 1977) and in lymphocytes (Schneider, 1976) and its cause is unknown.

How closely then do cells and tissues fixed for SEM resemble their in vivo counterparts? Surface structures, such as microvilli, blebs, and ruffles, have been observed by techniques other than conventional SEM. These structures have been seen on living cells with dark field microscopy (Willingham and Pastan, 1975). Scanning electron micrographs of cells prepared by freeze-drying instead of by conventional dehydration methods (Boyde et al., 1972), transmission electron micrographs, and whole cell replicas (Fisher and Cooper, 1967) also show these surface features. Nevertheless, cell shrinkage must alter surface morphology. When the volume of a nonelastic sphere decreases, but the surface area remains the same, the surface becomes wrinkled or convoluted. Likewise, cell surface structures, such as convolutions, blebs, additional microvilli, or lengthened existing microvilli, might appear when cells shrink. Convolutions

and blebs are not common characteristics of the HeLa cell surface (Figs. VII-1, p. 69; VII-2, p. 70; VII-5) and, thus probably do not form upon shrinkage. Microvilli contain microfilaments, and, therefore, formation of additional microvilli is unlikely, because it would require polymerization of microfilaments. In contrast, lengthening of existing microvilli, resulting from shrinkage is a feasible occurrence. Implicit in this idea is the existence of some rigid cell structure (cytoskeleton?) that prevents total collapse of the cell and limits the volume to which the cells can shrink. The similarity between the average diameter of the controls and the experimentally swelled cells (Figs. VII-1, p. 69; VII-2, p. 70; and Table VII-1, p. 71, lends support to the latter idea. Finally, other investigators have found that there is a minimum volume to which cells shrink in hypertonic medium (Dick, 1965; Cook, 1967).

SEM During the Cell Cycle

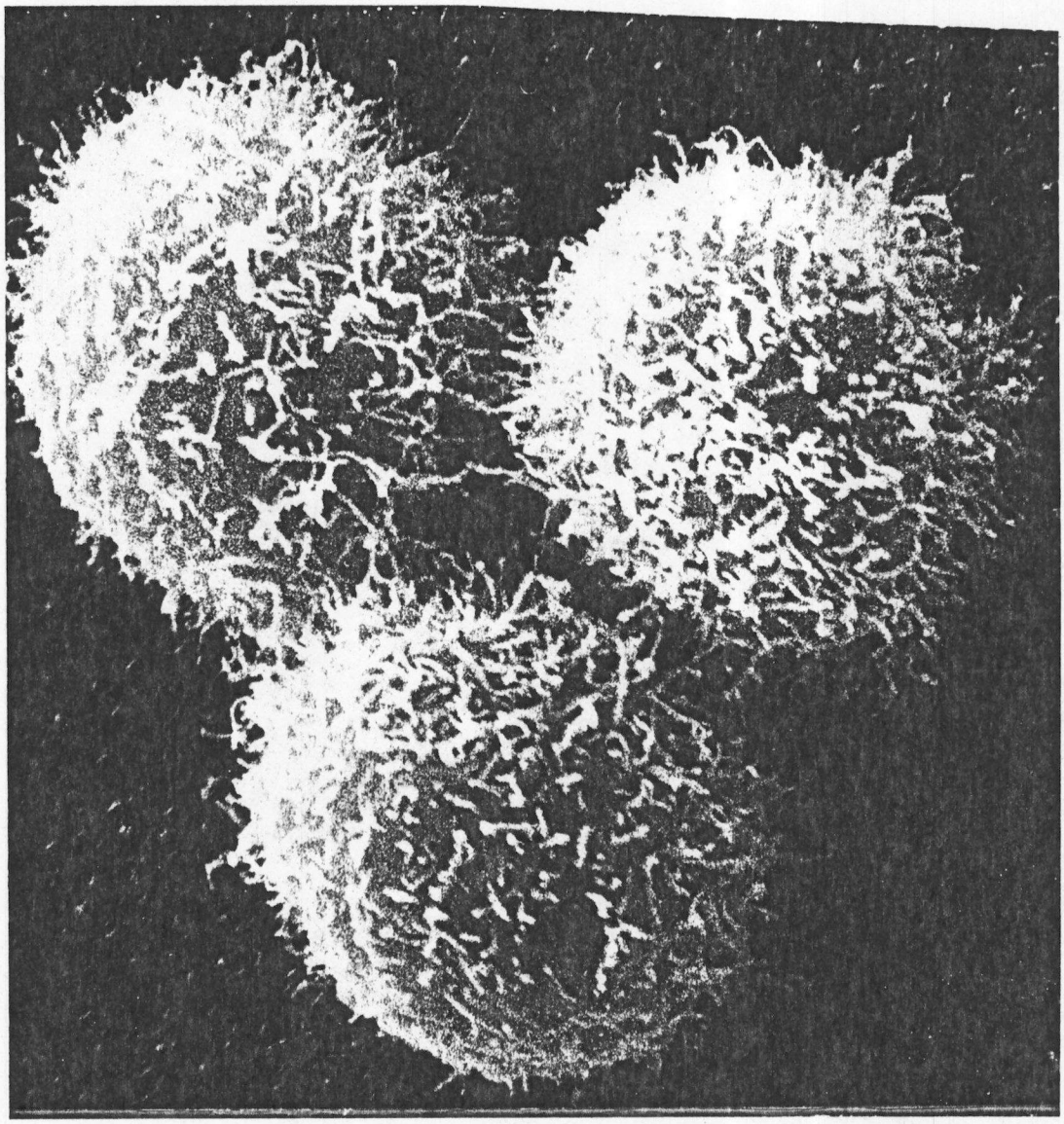
Given the above limitations in technique, my observations on synchronized HeLa S3 suspension cells indicated that very little variation in cell surface morphology occurs during the cell cycle (Fig. VII-5). Size differences, especially between cells in G_2 before division and G_1 after division, were evident (Fig. VII-5D). Cells in the DNA synthesis period (S) seemed to have characteristic patches of small blebs, but more experiments should be done to confirm this observation (Fig. VII-5A). Enlander and his coworkers (1975) also observed these patches of small blebs during late G_1 and early S in CHO cells.



A. S phase.

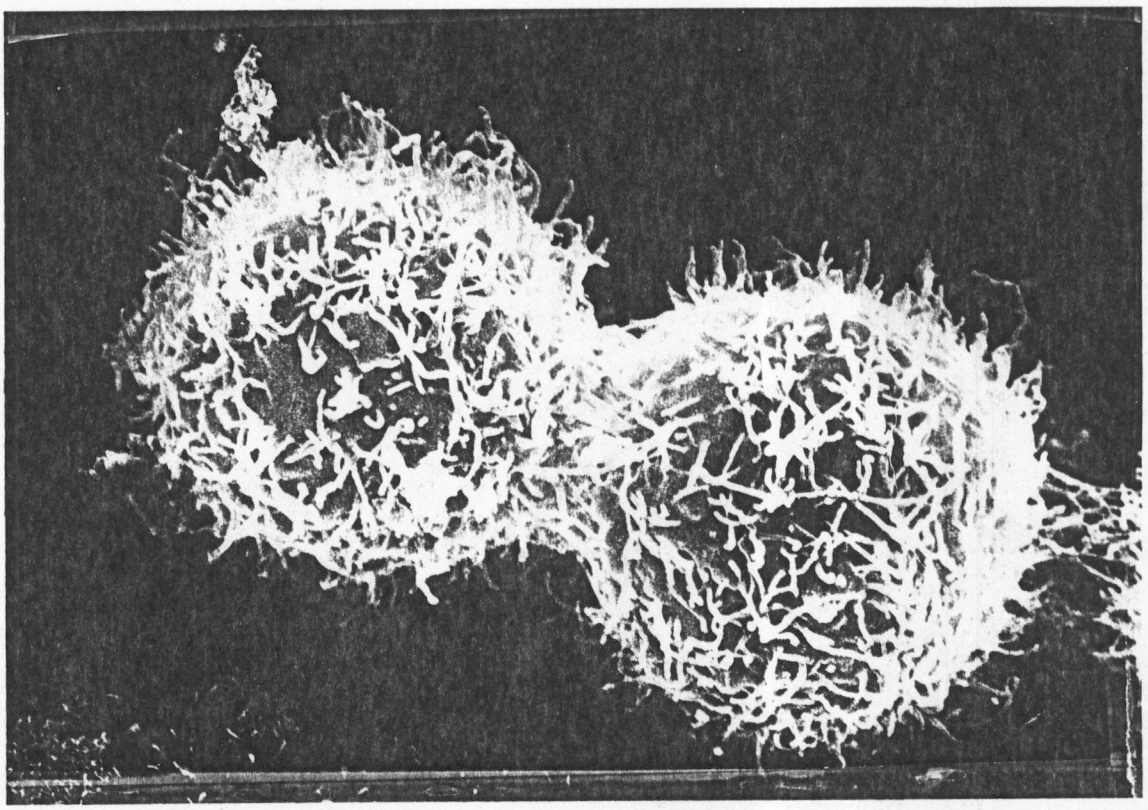
Fig. VII-5. Scanning electron micrographs of HeLa suspension cells at different stages of the cell cycle.

Cell suspensions were fixed and prepared for SEM as described in the text.



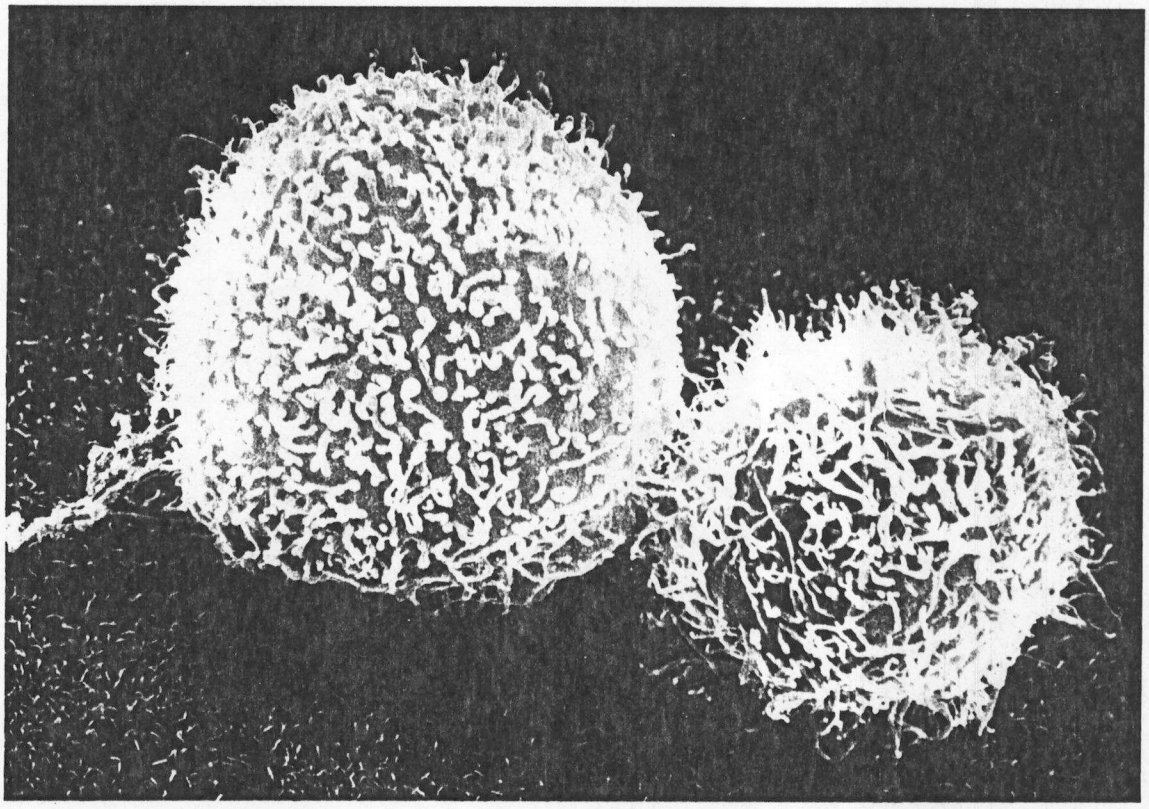
B. G_2 phase.

Fig. VII-5 (continued)



C. M phase.

Fig. VII-5 (continued)



D. During M. Large cell = G_2; small cell = early G_1.

Fig. VII-5 (continued)

CHAPTER VIII

SUMMARY AND OVERVIEW

In this study, several possible markers for nonadsorptive pinocytosis were evaluated. L-glucose, D-mannitol, and HRP were found to be unsatisfactory markers. In contrast, sucrose and inulin were evaluated as good pinocytic markers because they meet all the criteria listed in Chapter I. The uptake, intracellular fate, and efflux of sucrose, inulin, and internalized ouabain were then characterized. Uptakes of sucrose and inulin were classified as micropinocytosis because of their lack of dependence on cellular ATP and intact microfilaments. Ouabain influx was characterized as adsorptive macropinocytosis, because ouabain binds to the HeLa plasma membrane, and its uptake is sensitive to ATP depletion (Chapters I, III). In this chapter, some important unanswered questions will be discussed. Not all of them are directly related to the present study. The questions are concerned with the three aspects of endocytic marker turnover: influx, intracellular fate, and efflux.

An important question about pinocytic marker influx is related to general membrane structure. Are there specific membrane areas involved in endocytic uptake, or does this uptake occur with equal probability at all areas of the membrane? Tsan and Berlin (1974) found that several plasma membrane transport rates in alveolar macrophages remained unchanged after 35%-50% of the membrane had been

internalized by phagocytosis. This indicates that there are specific areas that function in phagocytosis but are devoid of sites for the transport functions measured. Their conclusions are supported by the observations of several other investigators (Griffin and Silverstein, 1973; DeCamilli et al, 1974; Dunham et al., 1974; R. Krogsrud, unpublished observations). In contrast, Steinman and Cohn (1976) think that phagocytosis involves a random, representative sampling of membrane in mouse macrophages. Their conclusion was based on the observation that three different plasma membrane labels appeared with equal frequency in an isolated phagolysosome fraction. Certain multivalent ligands, like Concanvalin A (Con A), which are taken up by adsorptive endocytosis, move laterally while attached to their receptors and aggregate prior to endocytosis. This aggregation, called "capping," and the subsequent endocytosis of the "caps" seem to occur in the polar region of lymphocytes (Taylor et al., 1971; De Petris and Raff, 1972). Thus, this region may be specialized for endocytosis.

How many pinosomes are formed per unit time, and how much surface area is involved in their formation? From the following assumptions: (1) a vesicle diameter range of 0.07-0.10 μm (Chapter I, III); (2) a cell volume of 2000 μ^3 (Chapter VII); (3) a surface area of 2300 μm^2, which is approximately threefold larger than that calculated for a 2000 μm^3 smooth sphere because of cell surface protrusions (Steinman et al., 1976); and (4) a micropinocytic rate of 0.05 pl/cell/hr (Chapter III), it can be calculated that HeLa suspension cells form

9.6×10^4 to 2.8×10^5 micropinosomes/hr (1590-4630 micropinosomes/min). The cell takes up an equivalent of 1.3 to 1.9 times its surface area by micropinocytosis/hr (2%-3%/min). Earlier experiments from our laboratory using a general protein labeling technique showed that the fastest detected turnover of any single HeLa membrane protein requires about 14 hr, a much slower rate than that obtained by the above calculations. Assuming a surface area of 2300 μM^2, all of which is involved in micropinocytosis, and a turnover rate of once per 14 hr, the average micropinosome diameter would be 1.5 μM, and the uptake rate would be 356 micropinosomes/hr. Such an uptake would be defined as macropinocytosis and would be sensitive to ATP depletion and cytochalasin B. However, sucrose and inulin uptakes are insensitive to these treatments (Chapter III). The apparent conflict in all of the above calculations can be reconciled by assuming that most membrane which is taken into the cell in endocytic vesicles is not degraded but is recycled to the surface. Steinman et al. (1976) found similar results and similarly concluded that there must be membrane recycling in L cells.

The next unanswered question is: What is the intracellular fate of endosomes? Storrie and Edelson (1977) have observed in CHO cells fusion of Con A-containing endosomes immediately after endocytosis but before the vesicles have moved away from the membrane into the cell. It is not known whether this fusion occurs in other cell types and with other markers. Transport of vesicles away from the membrane and into the cytoplasmic interior may involve binding of the vesicles

to microtubules and their subsequent transport away from the plasma membrane by saltatory motion (Freed and Lebowitz, 1970; Bhisey and Freed, 1971). Once in the cell interior, endosomes fuse with lysosomes and other endosomes (reviewed in Holtzman, 1976; Casley-Smith, 1969).

Why do endosomes fuse only with lysosomes and other endosomes and not with other cellular organelles? Several theories regarding fusion specificities are reviewed by Holtzman (1976). Proposed inducing factors for fusion include: (1) surface tension changes in localized membrane regions (Dingle, 1968, 1969); (2) Ca^{+2} and ATP displacement from the membranes (Poste and Allison, 1973); (3) chemical similarities between endosomal and lysosomal membranes (De Duve and Wattiaux, 1966); and (4) membrane lysolecithin content. Lysolecithin induces micelle formation, which in turn might facilitate fusion (Lucy, 1969; 1975).

Finally, how do nondigestible materials taken up by endocytosis escape from cells? Two possibilities were discussed in Chapter V. First, markers might slowly diffuse through the lysosomal membrane into the cytoplasm and then through the plasma membrane into the medium. Second, markers might stay contained inside vesicles, which randomly fuse with the plasma membrane and expel their contents by exocytosis into the medium. Presumably if exocytosis occurs, the plasma membrane which is contained in the vesicle, and which has not been degraded, would be recycled to the cell surface. It is possible then to test for exocytosis and membrane recycling by using

an adsorptive pinocytic marker, which binds tightly to a specific receptor on the plasma membrane and is not removed from the membrane by lysosomal hydrolysis. If exocytosis and recycling of intact plasma membrane occurs, the bound tracer could be followed sequentially from a purified plasma membrane fraction immediately after pulse binding, to an intracellular particulate cell fraction after endocytosis, and back to the plasma membrane fraction after exocytosis. De Duve and his associates (personal communication) have observed that IgG bound to the plasma membrane of cultured fibroblasts is cycled from the surface into the cell and back to the surface.

BIBLIOGRAPHY

BIBLIOGRAPHY

Allison, A. C., and Davies, P. (1974). Mechanisms of endocytosis and exocytosis in Transport at the Cellular Level. pp. 419-446. (Symp. Soc. Exp. Biol. XXVIII). Sleigh, M. A., and Jennings, D. H., eds. Cambridge University Press, Cambridge.

Amsterdam, A., Ohad, I., and Schramm, M. (1969). Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle. J. Cell Biol. 41: 753-773.

Baker, P. F., and Willis, J. S. (1972). Binding of the cardiac glycoside ouabain to intact cells. J. Physiol. 224: 441-462.

Becker, G., and Ashwood-Smith, M. J. (1973). Endocytosis in Chinese hamster fibroblasts. Exp. Cell Res. 82: 310-314.

Benassi, G. (1968). Effect of sucrose on the total acid phosphatase activity and the growth of LS cells. Exp. Cell Res. 50: 159-166.

Bennet, H. S. (1969). The cell surface in Handbook of Molecular Cytology. pp. 1261, 1294. Lima-da Faria, A., ed. North Holland Publishing Co., Amsterdam.

Bernacki, R. J., and Bosmann, H. B. (1971). The effects of sucrose and various salts on the growth and lysosomal enzyme activity of L5178Y cells. J. Cell Sci. 8: 399-406.

Bhisey, A. N., and Freed, J. J. (1971). Altered movement of endosomes in colchicine-treated cultured macrophages. Exp. Cell Res. 64: 430-438.

Boyde, A., Weiss, R. A., and Vesely, P. (1972). Scanning electron microscopy of cells in culture. Exp. Cell Res. 71: 313-324.

Bradford, W. D., Elchlepp, J. G., Arstila, A. U., Trump, B. F., and Kinney, T. D. (1969). Iron metabolism and cell membranes. Amer. J. Path. 56: 201-228.

Brunk, U. (1973). Distribution and shifts of indigested marker particles in residual bodies and other lysosomes. Exp. Cell Res. 79: 15-27.

Brunk, U., Bell, P., Colling, P., Forsby, N., and Fredriksson, B. A. (1975). SEM of in vitro cultivated cells, osmotic effects during fixation in Scanning Electron Microscopy. (Part I) Proceedings of the Eighth Annual Scanning Electron Microscope Symposium IIT

Research Institute, Chicago. pp. 379-386. Johari, O., and Corvin, I., eds.

Carr, I. (1973). The Macrophage: A Review of Ultrastructure and Function. Academic Press. New York.

Casley-Smith, J. R. (1969). Endocytosis: the different energy requirements for the uptake of particles by small and large vesicles into peritoneal macrophages. J. Micros. (Oxf.) 90: 15-30.

Chan, B. G., and Cain, T. C. (1966). A rapid quantitative method for routine determination of monosaccharades and oligosaccharides from plants by paper chromatography. J. Chromatog. 22: 95-101.

Cohen, S. R. (1969). A rapid, sensitive, semimicro gel filtration procedure for detecting and removing low molecular weight fragments from [^{3}H]- or [^{14}C]-labeled inulin. Anal. Biochem. 31: 539-544.

Cohn, Z. A. (1966). The regulation of pinocytosis in mouse macrophages I. Metabolic requirements as defined by the use of inhibitors. J. Exp. Med. 124: 557-571.

Cohn, Z. A. (1970). Endocytosis and intracellular digestion in Mononuclear Phagocytes. pp. 121-132. van Furth, R., ed. Oxford: Blackwell.

Cohn, Z. A., and Ehrenreich, B. A. (1969). The uptake, storage, and intracellular hydrolysis of carbohydrates by macrophages. J. Exp. Med. 129: 201-225.

Collard, J. G., and Temmink, J. H. M. (1975). Differences in density of Concanavalin A-binding sites due to differences in surface morphology of suspended normal and transformed 3T3 fibroblasts. J. Cell Sci. 19: 21-32.

Collard, J. G., and Temmink, J. H. M. (1976). Surface morphology and agglutinability with concanavalin A in normal and transformed murine fibroblasts. J. Cell Biol. 68: 101-112.

Cook, J. S. (1967). Nonsolvent water in human erythrocytes. J. Gen. Physiol. 50: 1311-1325.

Cook, J. S., and Brake, E. T. (1978). Uptake and fate of ouabain bound to HeLa cells in Drugs, Hormones, and Membranes. Bolis, L., Hoffman, J. F., and Straub, R. W., eds. Raven Press, New York, in press.

Cook, J. S., Vaughan, G. L., Proctor, W. R., and Brake, E. T. (1975). Interaction of two mechanisms regulating alkali cations in HeLa cells. J. Cell. Physiol. 86: 59-70.

Cook, J. S., Will, P. C., Proctor, W. R., and Brake, E. T. (1976). Turnover of ouabain-binding sites and plasma membrane proteins in HeLa cells in Biogenesis and Turnover of Membrane Macromolecules. pp. 15-36. Cook, J. S., ed. Raven Press, New York.

Creemers, J., and Jacques, P. J. (1971). Endocytic uptake and vesicular transport of injected horseradish peroxidase in the vacuolar apparatus of rat liver cells. Exp. Cell Res. 67: 188-203.

Davies, P., Allison, A. C., and Haswell, A. D. (1973a). The quantitative estimation of pinocytosis using radioactive colloidal gold. Biochem. Biophys. Res. Commun. 52: 627-634.

Davies, P., Allison, A. C., and Haswell, A. D. (1973b). Selective release of lysosomal hydrolases from phagocytic cells by cytochalasin B. Biochem. J. 134: 33-41.

Davies, P., Fox, R. I., Polyzonis, M., Allison, A. C., and Haswell, A. D. (1973c). The inhibition of phagocytosis and facilitation of exocytosis in rabbit PMN by cytochalasin B. Lab. Invest. 28: 16-22.

De Camilli, P., Peluchetti, D., and Meldolesi, J. (1974). Structural difference between luminal and lateral plasmalemma in pancreatic acinar cells. Nature 248: 245-247.

De Duve, C. (1963). in Ciba Symposium on Lysosomes. p. 126. De Reuck, A. V. S., and Cameron, M. P., eds. Little, Brown, and Co., Boston, Mass.

De Duve, C., and Wattiaux, R. (1966). Functions of lysosomes. in Ann. Rev. Physiol. vol. 28, pp. 435-492. Hall, V. E., ed. Annual Reviews, Inc., Palo Alto, California.

De Harven, E., Lampen, N., Sato, T., and Friend, C. (1973). Virology 51: 240-243.

De Petris, S., and Raff, M. C. (1972). Distribution of immunoglobulin on the surface of mouse lymphoid cells as determined by immunoferritin electron microscopy. Antibody-induced, temperature-dependent redistribution and its implications for membrane structure. Eur. J. Immunol. 2: 523-535.

Dick, D. A. T. (1965). Cell Water. Butterworths Scientific and Medical Publications, Washington, D. C.

Dingle, J. T. (1968). Vacuoles, vesicles, and lysosomes. Brit. Med. Bull. 24: 141-145.

Dingle, J. T. (1969). The extracellular secretion of lysosomal enzymes in Lysosomes in Biology and Pathology. vol. 2, pp. 421-436, Dingle, J. T., and Fell, H. B., eds. North Holland Publishing Co., Amsterdam.

Dolberg. D. S., Bassham, J. A., and Bissell, M. J. (1975). Selective inhibition of the facilitated mode of sugar uptake by cytochalasin B in cultured chick fibroblasts. Exp. Cell Res. 96: 129-317

Dunham, P. B., Goldstein, I. M., and Weissman, G. (1974). Potassium and amino acid transport in human leukocytes exposed to phagocytic stimuli. J. Cell Biol. 63: 215-226.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. Science 130: 432-437.

Ehrenreich, B. E., and Cohn, Z. A. (1967). The uptake and digestion of iodonated human serum albumen by macrophages in vitro. J. Exp. Med. 126: 941-958.

Ehrenreich, B. A., and Cohn, Z. A. (1968). Fate of hemoglobin pinocytized by macrophages in vitro. J. Cell. Biol. 38: 244-248.

Ehrenreich, B. A., and Cohn, Z. A. (1969). The fate of peptides pinocytosed by macrophages in vitro. J. Exp. Med. 129: 227-245.

Enlander, D. Tobey, R. A., and Scott, T. (1975). Cell cycle-dependent surface changes in Chinese hamster cells grown in suspension culture. Exp. Cell Res. 95: 396-404.

Everhart, L. P., and Rubin, R. W. (1974). Cyclic changes in the cell surface. II. The effect of cytochalasin B on the surface morphology of synchronized Chinese hamster ovary cells. J. Cell Biol. 60: 442-447.

Farquhar, M. G. (1969). Lysosome function in regulating secretion: disposal of secretory granules in cells of the anterior pituitary gland in Lysosomes in Biology and Pathology. vol. 2, pp. 462-482. Dingle, J., and Fell, H., eds. North Holland Publishing Co., Amsterdam.

Fell, H. B., and Dingle, J. T. (1966). Extracellular release of lysosomal enzymes in response to sucrose. Biochem. J. 98: 40P.

Fisher, H. W., and Cooper, T. W. (1967). Electron microscopic studies of the microvilli of HeLa cells. J. Cell Biol. 34: 569-576.

Freed, J. J., and Lebowitz, M. M. (1970). The association of a class of saltatory movements with microtubules in cultured cells. J. Cell Biol. 45: 334-354.

Gabathuler, M.-P., and Ryser, H. J.-P. (1969). The digestive function of lysosomes as studied by the turnover of injected foreign macromolecules. Proc. Roy. Soc. B. 173: 95-98.

Gordon, G. B., Miller, L. R., and Bensch, K. G. (1965). Studies on the intracellular digestive process in mammalian tissue culture cells. J. Cell Biol. 25: 41-55.

Graham, R. C., and Karnovsky, M. J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14: 291-302.

Griffin, F. M., and Silverstein, S. C. (1973). Segmental response of the macrophage plasma membrane to a phagocytic stimulus. J. Cell Biol. 59: 123a.

Gusnard, D., and Kirschner, R. H. (1977). Cell and organelle shrinkage during preparation for scanning electron microscopy: effects of fixation, dehydration and critical point drying. J. Microscopy 110: 51-57.

Hers, H. G. (1973). The concept of inborn lysosomal disease. in Lysosomes and Storage Diseases. pp. 147-171. Hers, H. G., and Van Hoof, eds. Academic Press, New York.

Holtzman, E. (1976). Lysosomes: A Survey, vol. 3. Alfert, M., Beermann, W., and Rudkin, G., eds. Springer-Verlag, New York.

Jacques, P. J. (1969). Endocytosis in Lysosomes in Biology and Pathology, vol. 2. pp. 395-420. Dingle, J. T., and Fell, H. B., eds. North Holland Publishing Co., Amsterdam.

Jamieson, J. D., and Palade, G. E. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. 34: 597-615.

Jennings, M. A., and Florey, F. R. S. (1967). An investigation of some properties of endothelum related to capillary permeability. Proc. Roy. Soc. B. 167: 39-63.

Knutton, S., Summer, M. C. B., and Pasternak, C. A. (1975). Role of microvilli in surface changes of synchronized P815Y mastocytoma cells. J. Cell Biol. 66: 568-576.

Knutton, S., Jackson, D., Graham, J. M., Micklem, K. J., and Pasternak, C. A. (1976). Microvilli and cell swelling. Nature 262: 52-53.

Kletzien, R. F., and Perdue, J. F. (1973). The inhibition of sugar transport in chick embryo fibroblasts by cytochalasin B-evidence for a membrane specific effect. J. Biol. Chem. 248: 711-719.

Kregenow, F. M. (1971). The response of duck erythrocytes to hypertonic media. J. Gen. Physiol. 58: 396-412.

Lamb, J. F., and MacKinnon, M. G. A. (1971). Effect of ouabain and metabolic inhibitors on the Na and K movements and nucleotide contents of L cells. J. Physiol. 213: 665-682.

Lamb, J. F., and McCall, D. (1972). Effect of prolonged ouabain treatment of Na, K, Cl, and Ca concentrations and fluxes in cultured human cells. J. Physiol. 225: 599-617.

Kletzien, R. F., and Perdue, J. F. (1975). Induction of sugar transport in chick embryo fibroblasts by hexose starvation. J. Biol. Chem. 250: 593-600.

Lewis, W. H. (1931). Pinocytosis. Bull. Johns Hopkins Hosp. 49: 17-27.

Lin, P. S., Wallach, D. F. H., and Tsai, S. (1973). Temperature-induced variations in the surface topology of cultured lymphocytes are revealed by scanning electron microscopy. Proc. Natl. Acad. Sci. U.S.A. 70: 2492-2496.

Lucy, J. A. (1969). Lysosomal membrane in Lysosomes in Biology and Pathology. vol. 2, pp. 313-341. Dingle, J. T., and Fell, H. B., eds. North Holland Publishing Co., Amsterdam.

Lucy, J. A. (1975). The fusion of cell membranes in Cell Membranes Biochemistry, Cell Biology, and Pathology. pp. 75-83. Weissman, G., and Claiborne, R., eds. HP Publishing Co., New York.

Masur, S. K., Holtzman, E., and Walter, R. (1972). Hormone stimulated exocytosis in the toad urinary bladder. Some possible implications for turnover of surface membrane. J. Cell Biol. 52: 211-219.

Mazia, D., Schatten, G., and Sale, W. (1975). Adhesion of cells to surfaces coated with polylysine. J. Cell Biol. 66: 198-200.

Molday, R. S., Dreyer, W. J., Renbaum, A., and Yen, S. P. S. (1975). New immunolatex spheres: visual markers of antigens on lymphocytes for scanning electron microscopy. J. Cell Biol. 64: 75-88.

Nemanic, M. K., Carter, D. P., Pitelka, D. R., and Wofsy, L. (1975). Hapten-sandwich labelling. II. Immunospecific attachment of cell surface markers suitable for scanning electron microscopy. J. Cell Biol. 64: 311-321.

Norman, T. C. (1976). Neurosecretion by exocytosis. in Int. Rev. Cytol. vol. 46, pp. 1-77. Bourne, G. H., and Danielli, J. F., eds. Academic Press, New York.

Novikoff, A. B. (1973) Lysosomes: A personal account in Lysosomes and Storage Diseases. pp. 1-41. Hers, H. G., and van Hoff, F., eds. Academic Press, New York.

Nyberg, E., and Dingle, J. T. (1970). Endocytosis of sucrose and other sugars by cells in culture. Exp. Cell Res. 63: 43-52.

Orci, L., Perrelet, A., and Friend, D. S. (1977). Freeze-fracture of membrane fusions during exocytosis in pancreatic B-cells. J. Cell Biol. 75: 23-30.

Palade, G. E. (1959). Functional changes in the structure of cell components in Subcellular Particles, pp. 64-83. Hayaski, T., ed. The Ronald Press Co., New York.

Pedersen, P. L., Greenawalt, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Soper, J. W., and Bustamante, E. (1977). Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues in Methods in Cell Biology, vol. 18, Prescott, D. A., ed. Academic Press, New York, in press.

Pelletier, G., Peillon, F., and Vila-Porcile, E. (1971). An ultrastructural study of sites of granule extrusion in the rat. Z. Zellforsch. Mikrosk. Anat. 115: 501-507.

Petersen, D. F., and Anderson, E. C. (1964). Quantity production of synchronized mammalian cells in suspension cultures. Nature (Lond.) 203: 642-645.

Petitpierre-Gabathuler, M.-P., and Ryser, H. J.-P. (1975). Cellular uptake of soluble and aggregated ferritin: distinction between pinocytosis and phagocytosis. J. Cell Sci. 19: 141-156.

Porter, K., Prescott, D., and Frye, J. (1973). Changes in surface morphology of Chinese hamster ovary cells during the cell cycle. J. Cell Biol. 57: 815-836.

Poste, G., and Allison, A. C. (1973). Membrane fusion. Biochem. Biophys. Acta 300: 421-465.

Poste, G., and Papahadjopoulos, D. (1976). Lipid vesicles as carriers for introducing materials into cultured cells: influence of vesicle lipid composition on mechanism(s) of vesicle incorporation into cells. Proc. Natl. Acad. Sci. U.S.A. 73: 1603-1607.

Proctor, W. R. (1974). Membrane protein turnover and specific membrane enzyme activities during the HeLa cell cycle. Ph. D. Dissertation. The University of Tennessee, Knoxville.

Puck, T. T., Marcus, P. I., and Cieiura, S. J. (1956). Clonal growth of mammalian cells in vitro (growth characteristics of colonies from single HeLa cells with and without a "feeder" layer). J. Exp. Med. 103: 273-284.

Quintartx, J., Bartholeyns, J., and Baudhuin, P. (1976). Subcellular distribution of horseradish peroxidase incorporated by hepatoma tissue cells in culture. J. Cell Biol. 70: 134a.

Renner, E. D., Plagemann, P. G. W., and Bernlohr, R. W. (1972). Permeation of glucose by simple and facilitated diffusion by Novikoff rat hepatoma cells in suspension culture and its relationship to glucose metabolism. J. Biol. Chem. 247: 5765-5776.

Ricketts, T. R. (1971). Endocytosis in Tetrahymena pyriformis. Exp. Cell Res. 66: 49-58.

Rosenberg, H. M., Shank, B. B., and Gregg, E. C. (1972). Volume changes of mammalian cells subjected to hypotonic solutions in vitro: evidence for the requirement of a sodium pump for the shrinking phase. J. Cell. Physiol. 80: 23-32.

Ryser, H. J.-P. (1967a). Studies on protein uptake by isolated tumor cells. III. Apparent stimulations due to pH, hypertonicity, polycations, or dehydration and their relation to the enhanced penetration of infectious nucleic acids. J. Cell Biol. 32: 737-750.

Ryser, H. J.-P. (1967b). A membrane effect of basic polymers dependent on molecular size. Nature (Lond.) 215: 934-936.

Ryser, H. J.-P., and Hancock, R. (1965). Histones and basic polyamino acids stimulate the uptake of albumin by tumor cells in culture. Science 150: 501-503.

Salter, D. W. (1977). Hexose transport and metabolism in cultured cells. Ph. D. Dissertation. The University of Tennessee, Knoxville.

Sanders, S. K., Alexander, E. L., and Braylan, R. C. (1975). A high-yield technique for preparing cells fixed in suspension for scanning electron microscopy. J. Cell Biol. 67: 476-480.

Schneider, G. B. (1976). The effects of preparation procedures for scanning electron microscopy on the size of isolated lymphocytes. Am. J. Anat. 146: 93-100.

Schneider, E. L., Stanbridge, E. J., and Epstein, C. J. (1974). Incorporation of ^{3}H-uridine and ^{3}H-uracil into RNA—a simple technique for the detection of mycoplasma contamination of cultured cells. Exp. Cell Res. 84: 311-318.

Seljelid, R., Silverstein, S. C., and Cohn, Z. A. (1973). The effect of poly-L-lysine on the uptake of reovirus double-stranded RNA in macrophages in vitro. J. Cell Biol. 57: 484-498.

Shank, B. B., Rosenberg, H. M., and Horowitz, C. (1973). Ionic basis of volume regulation in mammalian cells following osmotic shock. J. Cell. Physiol. 82: 257-266.

Steinman, R. M., and Cohn, Z. A. (1972). The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. J. Cell Biol. 55: 186-204.

Steinman, R. M., and Cohn, Z. A. (1976). Endocytosis and the vacuolar apparatus in Biogenesis and Turnover of Membrane Macromolecules, pp. 1-14. Cook, J. S., ed. Raven Press, New York.

Steinman, R. M., Silver, J. M., and Cohn, Z. A. (1974). Pinocytosis in fibroblasts—quantitative studies in vitro. J. Cell Biol. 63: 949-969.

Steinman, R. M., Brodie, S. E., and Cohn, Z. A. (1976). Membrane flow during pinocytosis: a stereologic analysis. J. Cell Biol. 68: 665-687.

Storrie, B., and Edelson, P. J. (1977). Distribution of concanavalin A in fibroblasts: direct endocytosis versus surface capping. Cell 11: 707-717.

Stossel, T. P., Mason, R. J., Hartwig, J., and Vaughan, M. (1972). Quantitative studies of phagocytosis by polymorphonuclear leucocytes: use of emulsions to measure the initial rate of phagocytosis. J. Clin. Invest. 51: 615-624.

Strehler, B. L. (1974). Adenosine-5'-triphosphate and creatine phosphate determination with luciferase in Methods of Enzymatic Analysis, 2nd edition, vol. 4, pp. 2112-2126. Bergmeyer, H. U., ed. Academic Press, New York.

Taylor, R. B., Philip, W., Duffus, H., Raff, M. C., De Petris, S. (1971). Redistribution and pinocytosis of lymphocyte surface

immunoglobulin molecules induced by anti-immunoglobulin antibody. Nat. New Biol. 233: 225-229.

Tsan, M. F., and Berlin, R. D. (1971). Effect of phagocytosis on membrane transport of nonelectrolytes. J. Exp. Med. 134: 1016-1035.

Vernon-Roberts, B. (1972). The Macrophage. Cambridge University Press, Cambridge.

Wagner, R., Rosenberg, M., and Estensen, R. (1971). Endocytosis in Chang liver cells. Quantitation by sucrose -^{3}H uptake and inhibition by cytochalasin B. J. Cell Biol. 50: 804-817.

Wallace, R. A., and Ho, T. (1972). Protein incorporation by isolated amphibian oocytes. II. A survey of inhibitors. J. Exp. Zool. 181: 303-317.

Wattiaux, R., Wibo, M., and Baudhuin, P. (1963). Influence of the injection of Triton WR-1339 on the properties of rat-liver lysosomes in Ciba Foundation Symposium on Lysosomes, pp. 176-200. De Reuck, A. V. S., and Cameron, M. P., eds. Little Brown, Boston.

Weber, M. J. (1973). Hexose transport in normal and in Rous sarcoma virus-transformed cells. J. Biol. Chem. 248: 2978-2983.

Will, P. C., Longworth, J. W., Brake, E. T., and Cook, J. S. (1977). Analysis of intracellular drug (ouabain) sequestration as a mechanism of detoxification. Mol. Pharmacol. 13: 161-171.

Williams, K. E., Kidston, E. M., Beck, F., and Lloyd, J. B. (1975a). Quantitative studies of pinocytosis. I. Kinetics of uptake of [^{125}I]polyvinylpyrrolidone by rat yolk sac cultures in vitro. J. Cell Biol. 64: 113-122.

Williams, K. E. M., Kidston, E., Beck, F., and Lloyd, J. B. (1975b). Quantitative studies of pinocytosis. II. Kinetics of protein uptake and digestion by rat yolk sac cultured in vitro. J. Cell Biol. 64: 123-134.

Willingham, M. C., and Pastan, I. (1975). Cyclic AMP modulates microvillus formation and agglutionability in transformed and normal mouse fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 72: 1263-1267.

Wills, E. J., Davies, P., Allison, A. C., and Haswell, A. D. (1972). The failure of cytochalasin B to inhibit pinocytosis by macrophages. Nat. New Biol. 240: 58-60.

VITA

Emily Tate Brake was born in Knoxville, Tennessee, on January 20, 1946. She attended elementary school in Knoxville and was graduated from Bearden High School in June of 1964. She entered the University of Tennessee in September of 1964 and received a Bachelor of Science degree in Microbiology in March of 1969.

In August of 1969, she was employed by the University of Tennessee Memorial Research Center and Hospital where she was a Research Assistant until September of 1971.

In October of 1971, she was employed by Oak Ridge National Laboratory, Biology Division, Oak Ridge, Tennessee. She began work on a Masters Degree in September of 1972 and received the Master of Science degree with a major in Biomedical Sciences in December of of 1977.

The author is a member of Phi Beta Kappa, Sigma Xi, American Association for the Advancement of Science, and The American Society for Cell Biology.

She is married to Richard Joseph Brake of Oklahoma City.