To the Graduate Council:

I am submitting herewith a dissertation written by Tae In Ahn entitled "Comparisons of Cytochemical and Biochemical Characteristics of Phagosomes and Symbiotic Vacuoles in xD Strain of Amoeba proteus." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

Kwang W. Jeon, Major Professor

We have read this dissertation
and recommend its acceptance:

Leaf Huang

Mary Ann Handel

Lowell C. Fraser

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

COMPARISONS OF CYTOCHEMICAL AND BIOCHEMICAL CHARACTERISTICS OF PHAGOSOMES AND SYMBIOTIC VACUOLES IN XD STRAIN OF *AMOEBA PROTEUS*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Tae In Ahn

March 1981

3049528

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my committee chairman, Dr. Kwang W. Jeon, for his support and guidance towards the completion of this dissertation. I also thank the members of my committee, Drs. Ronald C. Fraser, Mary Ann Handel, Leaf Huang, and Gary L. Whitson, for their advice and helpful discussion. Special thanks are due to Dr Huang for his many suggestions and encouragements during the course of my work.

This study was supported by Dr Jeon's grant for the study of the endosymbiosis in amoebae. I am also grateful to the Zoology Department at the University of Tennessee for awarding me a teaching assistantship since September 1979.

I thank Dr. K. H. Kim for his kind help in the preparation of this dissertation. I express my appreciation to my mother for her prayers and her everlasting encouragement.

This dissertation is dedicated to my wife Sang Sook for her support and understanding.

ABSTRACT

Cytochemical and biochemical characteristics of the membranes of phagosomes and symbiotic vacuoles were compared to detect possible functional and structural differences between the two vacuole systems. Fluorescence microscopy of acridine orange-labeled cells and electron microscopy of ferritin-labeled xD amoebae showed that the absence of acid phosphatase activity in the symbiotic vacuole was due to the inhibition of lysosomal fusion.

Pure fractions of the plasmalemma, phagosomes, and symbiotic vacuoles were obtained by a newly developed procedure. The presence of functional markers was studied by enzyme cytochemistry and assay of marker enzymes in the subcellular fractions. None of the marker enzymes of the plasma membranes, i.e., (Na^+-K^+)-ATPase, 5'-NTPase, or alkaline phosphodiesterase, was detected, however.

Modification of the plasmalemma following phagocytosis was examined by electron microscopy. The glycocalyx was completely lost within 2 hours of endocytosis. The loss of glycocalyx and the modification of macromolecular composition were also shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Changes in the banding patterns of proteins and glycoproteins were observable more distinctly after iodination from the cytoplasmic surface of purified phagosomes by lactoperoxidase. The cytoplasmic surface of the purified symbiotic vacuoles and the phagosomes was iodinated with I-125 by lactoperoxidase. The presence of a unique protein in the membranes of symbiotic vacuoles

was detected by gel radioautography. The possible role of the unique protein is discussed with respect to the maintenance of endocellular symbiosis and the inhibition of lysosomal fusion.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	7
	1. Materials	7
	2. Cell Culture	8
	3. Subcellular Fractionation	9
	4. Fluorescence Microscopy of Acridine Orange-Labeled Cells	18
	5. Electron Microscopy and Enzyme Cytochemistry	18
	6. Assay of Membrane Marker Enzymes	21
	7. Iodination of Subcellular Fractions	23
	8. SDS Polyacrylamide Gel Electrophoresis and Gel Radioautography	25
III.	RESULTS	27
	1. Lysosomal Fusion	27
	2. Phagocytosis and Changes in Membrane Morphology	33
	3. Cytochemistry of Marker Enzymes	33
	4. Purity and Morphology of Subcellular Fractions	36
	5. Iodination of Subcellular Fractions	53
	6. SDS PAGE of Subcellular Fractions	53
	7. Assay of Marker Enzymes	64
IV.	DISCUSSION	72
BIBLIOGRAPHY		84
APPENDICES		92
	APPENDIX I	93
	APPENDIX II	94
VITA		96

LIST OF FIGURES

FIGURE PAGE

1. A Procedure for Isolating Cell Membranes - - - - - - - - - - - - 10
2. A Procedure for Isolating Phagosomes - - - - - - - - - - - - - - 13
3. A Procedure for Isolating Symbiotic Vacuoles - - - - - - - - - 15
4. A Procedure for Isolating Symbiotic Bacteria - - - - - - - - - 17
5. Fusion between Acridine Orange-Labeled Secondary Lysosomes and Phagosomes - - - - - - - - - - - - - - - 28
6. Electron Micrograph Showing Lysosomal Fusion in an xD Amoeba - 31
7. Morphology of Glycocalyx of the Cell Surface Following Endocytosis and Digestion - - - - - - - - - - - - - 34
8. Enzyme Cytochemistry of Acid Phosphatase in the xD Amoeba - 37
9. Enzyme Cytochemistry of Glucose 6-Phosphatase in the xD Amoeba - 39
10. Morphology of Cellular Organelles before Isolation - - - - - - 42
11. Purified Plasmalemma at a Low Magnification - - - - - - - - - 44
12. Ultrastructure of Purified Plasmalemma - - - - - - - - - - - - 46
13. Electron Micrographs of Purified Phagosomes Containing Latex Beads - 48
14. Morphology of the Purified Symbiotic Vacuoles - - - - - - - - 51
15. SDS PAGE of Purified Plasmalemma and Phagosomes - - - - - - - 55
16. Gel Radioautograms of the Plasmalemma and Phagosomes - - - - - 57
17. SDS PAGE of Purified Plasmalemma, Symbiotic Vacuoles and Bacteria - 60
18. Gel Radioautograms of Plasmalemma, Phagosomes, Symbiotic Vacuoles and Bacteria - - - - - - - - - - - - - - - 62

FIGURE PAGE

19. A pH Profile of Marker Enzymes in xD Amoebae - - - - - - - - - - 65

20. Inhibition of AcPase of xD Amoebae - - - - - - - - - - - - - - - - 67

21. Inhibition of ATPase of xD Amoebae by Ouabain - - - - - - - - 70

22. The Effect of Ionic Composition on ATPase Activity - 70

CHAPTER 1

INTRODUCTION

One of the important mechanisms for an organism to become a persistent and organismically dependent endosymbiont is the endosymbiont's ability to avoid of destruction or ejection by the host cell (Jeon & Jeon, 1976; Taylor, 1976). In contrast, the digestion and intracellular killing of microorganisms is one of the central mechanisms in the host defense (Sbarra et al., 1971; Klebanoff, 1975; Quie, 1975; Johnston & Lehmeyer, 1977; Peterson et al., 1977).

The phagosome which contains an acid milieu pH between 4 and 5, which is a collection of some 40 or more digestive enzymes, is capable of extensively dismantling a large variety of complex materials by the concerted or sequential action of the enzymes (DeDuve et al., 1974). The failure or alteration of the membrane fusion reaction may result in pathological dysfunction (Poste & Allison, 1973). Digestion is clearly incompatible with the survival and multiplication of virulent bacteria, however (Armstrong & Hart, 1971).

Ordinarily, a variety of infectious agents or endosymbionts enter their host cells within endocytic vacuoles but have different ultimate intracellular destinations. Several obligate intracellular bacterial pathogens (e.g., tubercle bacilli, listeria monocytogenes) are sequestered within host cell lysosomes where they replicate, eventually destroying the host cell. Chlamydia and Toxoplasma utilize the vacuolar apparatus in another way. They enter cells within endocytic vacuoles,

but prevent the fusion of their vacuoles with host-cell lysosomes (Fris, 1972; Jones & Hirsch, 1972a,b; Hirsch et al., 1974). Presumably their capacity to inhibit lysosome-phagosome fusion requires continued production of metabolites or synthetic products by these organisms, since dead Chlamydia and Toxoplasma are digested in lysosomes (Jones & Hirsch, 1972b; Hirsch et al., 1974). Similar observations were also made in the algal symbionts of invertebrates (Karakashian, 1975; Muscatine et al., 1975). The endocytosed algal symbionts appear to interfere with the host digestive process and gradually segregate into their individual vacuoles to prevent their own digestion either by inhibition of the host's digestive enzymes or by the prevention of lysosomal fusion.

Thus, the endocellular parasites or endosymbionts enter the host cells within the endocytic vacuoles. After gaining entrance, they should have the fitness trait for intracellular survival (Moulder, 1974, 1979) by resisting digestion and killing (Smith, 1968, 1977; Allen, 1969; Chang & Dwyer, 1976, 1978), or by preventing the release of lysosomal enzymes (Allen, 1969; Armstrong & Hart, 1971; Fris, 1972; Jones & Hirsch, 1972b). Such a preventive mechanism appears to be specific to each symbiotic or parasitic relationship (Jeon & Jeon, 1976). Various types of ingested organisms are known to be more or less specifically sensitive to a certain component enzyme of lysosomes (Allen, 1969). A loss of lysosomal enzymes has been noticed in heavily infected cells in paratuberculosis of sheep and in murine leprosy

(Allen, 1969). Finally, to establish a permanent endocellular association, the vacuoles containing the invading organism should remain within the cytoplasm without ejection by the host. Most of the studies on the interaction between lysosomes and the vacuoles containing the endoparasites or endosymbionts have been done by cytochemistry of acid phosphatase or by use of electron-dense markers to label the secondary lysosomes. In many instances the vacuoles containing parasites or symbionts have shown the absence of fusion with lysosomes, but no other mechanism for physiological or biochemical protection has been found. After the endocytic vacuoles have been formed, they pass through a series of coalescences and subdivisions, involving a series of membrane-fusion sequences between the vacuoles and lysosomes (Jacques, 1969; Chapman-Andresen, 1973).

The organismic association occurs via the vacuole membrane that is originally derived from the host cell membrane. If so, the membrane becomes greatly altered as it is integrated into the structure and economy of the infiltrating organisms (Stokes, 1974; Trager, 1974). Non-identity of the infected cell membrane with non-infected cell membrane has been shown by immunological methods (Trager, 1974) and by ^{14}C-amino acid labeling studies (Stokes, 1973).

Very little is known about the molecular dynamics of membrane fusion in the phagolysosomes (Dingle, 1968; Jacques, 1969; Silverstein et al., 1977). Our present knowledge of the evolution of phagosomes is too slight and is limited to the observation of the coalescence or

budding of phagosomes and to the alteration of mucus layer (Jacques, 1969). The vacuoles and lysosomes are all bounded by a unit membrane. The manner in which the composition and physical structure of the membrane may influence the interaction of the organelles with one another and with other cytoplasmic structure, is not well understood (Dingle, 1968). However, it seems likely that the control of cellular digestive function, in both normal physiology and in pathology, may largely depend on alteration in the physical properties of the plasma membrane and the organelles. Some of the biochemical studies of membrane lipids of the digestive apparatus have shown no qualitative differences among mitochondria, microsomes, and plasma membranes of rat liver (Dingle, 1968). It is almost impossible to draw conclusions concerning the relationship between the lipid composition and function. The associated proteins or glycoproteins have been suggested to be different in composition or amount (Dingle, 1968). Phagosomes containing different particles have different arrangements of membrane components, and show varying efficiencies of lysosomal fusion (Rikihisa & Mizuno, 1978).

As stated by Oates and Touster (1976), the lack of an _in vitro_ system has precluded any straight-forward biochemical study of vacuole fusion and has severely limited the progress in our understanding of this process. In the last few years they have developed a model system for studying fusion of phagolysosomes _in vitro_ using _Acanthamoeba_ (Oates & Touster, 1976, 1978, 1980).

Among many different phagocytic cells or free-living amoeboid single-cell organisms, the xD strain of Amoeba proteus is an excellent model system for the study of the preventive mechanism of lysosomal fusion in pathology and membrane fusion of phagolysosomes. The X-bacteria have been transformed from harmful parasites (Jeon & Lorch, 1967) to obligate symbionts. Studies in the last several years (Jeon, 1972; Jeon & Jeon, 1976; Jeon & Hah, 1977; Jeon & Ahn, 1978) have shown that this amoeba is in symbiotic association with the bacteria which are enclosed in membrane-bounded vesicles. In the study of acid phosphatase cytochemistry, the symbiotic vesicles did not show the enzyme activity (Jeon & Jeon, 1976). The isolated endosymbiotic bacteria can be introduced to the nonsymbiotic strain (D) of amoeba by induced phagocytosis (Ahn & Jeon, 1979). The ingested bacteria are segregated from other food material of the phagosome, and the separated vesicles develop to symbiotic vesicles. Amoebae are known to be rich in lysosomal enzymes (Chapman-Andresen, 1973), and they destroy any exogeneous bacteria in the cytoplasm or in the food vacuoles (Savanat & Pavillard, 1964; Casley-Smith & Savanat, 1966).

The obligate symbiotic amoeba (xD) has shown many characteristics which are suitable for the study of phagolysosomal interactions and of the establishment of endocellular organismic association. For example, it is a large free-living protozoan whose cell size is about 100 μm in diameter; it is highly phagocytic and rich in lysosomal enzymes; within the cytoplasm it can have both lysosome-fusible and non-fusible

vacuoles that are originaly derived by phagocytosis; the symbionts do not overpopulate to exceed the host's carrying capacity.

A study of xD amoebae is significant not only in understanding the compatibility mechanism of the symbiotic association, but also in understanding the mechanism of phagolysosomal fusion. Utilization of this system will be good for the study of lysosomal fusion in vitro as well (cf. Raz & Goldman, 1974; Oates & Touster, 1976, 1978).

The present study was initiated to examine structural and functional modifications in the membranes of food vacuoles during their change to symbiotic vacuoles. The difference between these two vacuole systems was examined after labeling the phagosomes of xD amoebae with non-digestible polystyrene latex beads. Fusion of lysosomes in vivo was compared after labeling the secondary lysosomes with fluorescent acridine orange or with electron-dense markers. The differences in membrane markers were examined by enzyme cytochemistry and by enzyme assay of the subcellular fractions. Protein composition of the vacuole preparations was compared by SDS-polyacrylamide gel electrophoresis. The cytoplasmic side of the vacuole membrane was labeled with I-125 by lactoperoxidase. The banding pattern of the labeled protein was compared by gel radioautography.

CHAPTER II

MATERIALS AND METHODS

1. Materials

The enzyme substrates, adenosine 5'-triphosphate (disodium salt, grade II), D-glucose 6-phosphate (Sigma grade), adenosine 5'-monophosphoric acid (type II), p-nitrophenylphosphate (disodium), thiaminepyrophosphate, p-nitrophenyl-N-acetyl-β-D-glucosaminide (grade III), and thymidine 5'-monophospho-p-nitrophenylester, were purchased from Sigma Chemical Co. (St Louis, MO.). Acid molibdate solution for inorganic phosphate assay, phenol reagent for protein assay, ferritin (type I), acridine orange, ouabain, and sucrose (grade I) for density gradient were from Sigma Chemical Co. Nylon screens were from Tobler, Ernst and Traber, Inc. (Elmsford, N.Y). Carrier-free iodine-125 (specific activity 13-17 mCi/μg, 100 mCi/ml) was from Amersham Corporation (Arlington Height, IL). Lactoperoxidase (B grade, 15 I.U./mg protein) was from CalBiochem (La Jolla, CA.). SDS (electrophoresis purity grade) was from BioRad Laboratories (Richmond, CA.). Acrylamide and N,N'-methylene-bisacrylamide (ultrapure electrophoresis grade) were from Polyscience, Inc. (Warrington, PA.). Mercaptoethanol, X-Omat R film, developer and fixer for the X-ray film were from Eastman Kodak Co. (Rochester, NY). Buffer components and other inorganic chemicals (all reagent grade) were purchased from Fisher Chemical Co. (Pittsburgh, PA.).

2. Cell Culture

AMOEBA An infected varient strain of Amoeba proteus, xD(Jeon & Lorch, 1967) was cultured in a modified Chalkley's medium (Jeon & Jeon, 1975) (Appendix I) with axenically cultured Tetrahymena pyriformis as food organisms. The cells were grown in Pyrex baking dishes (34x22x4 cm) with medium to a depth of 2 cm. To change dishes on every feeding day, old culture medium was aspirated, and the scum, molds and floating amoebae were decanted to another dish. The cells that attached to the bottom of the dish were transfered to a clean dish with fresh medium. Then the amoebae were fed with freshly harvested Tetrahymena (40 ml of $1.3x10^5$ Tetrahymena / ml per dish containing $4x10^5$ amoebae). The amoebae were fed every day or every other day depending on the need of amoebae. Culture dishes were changed every other day.

TETRAHYMENA Tetrahymena pyriformis (GL strain) was cultured axenically in the medium described by Goldstein and Ko(1976). The culture medium (Appendix II) consisted of a mixture of salt and vitamin solutions, liver concentrate, and proteose peptone. The stock solution of salts and vitamins was prepared and kept at -20 °C. The frozen stock solution was good up to a year in storage. After dissolving 40 gm of proteose peptone and 4 gm of liver concentrate in distilled water, 20 ml of the frozen salt and vitamin mixture were added and filled up to 1000 ml. This medium was divided in 500-ml flasks with 100 ml each or 1000-ml flasks with 200 ml each, and was autoclaved for 15 min at 121 °C

at 15 psi above atmospheric pressure. After inoculating 5 ml of full-grown Tetrahymena culture, the cells were grown for 2 days at room temperature in a shaker bath (Blue M Electric Co., Blue Island, IL) set at 100 rpm. For harvesting, Chalkley's solution equal to one-half volume of the culture was added and the culture was left shaking for 10 min to minimize the osmotic shock. Tetrahymena were harvested by centrifugation for 1 min at 350 g and washed twice in Chalkley's medium. The yield was about 6 ml of packed Tetrahymena from 200-ml culture. Then the packed Tetrahymena were resuspended in 150 times volume of the Chalkley's. This preparation contained about 1.3×10^5 Tetrahymena /ml.

3. Subcellular Fractionation

All procedures for the subcellular fractionation were carried at 4 °C, unless otherwise noted. All sucrose solutions were prepared by w/w in TH buffer (Tris-HCl buffer, 0.02 M pH 7.4).

PLASMALEMMA With the conventional methods for isolating cell membranes, i.e., aqueous two-phase system (Brunette & Till, 1971), sucrose step-gradient method (O'Neil, 1964), and glycerol step-gradient method (Allen et al., 1974), the membrane yield was extremely low and most of the large membrane fragments were pelleted at the bottom of the tube together with cell nuclei and crystals even in 60 % sucrose. The following procedure (Fig. 1) was developed and used routinely to prepare plasmalemma. Mass-cultured amoebae were starved for 3 days with

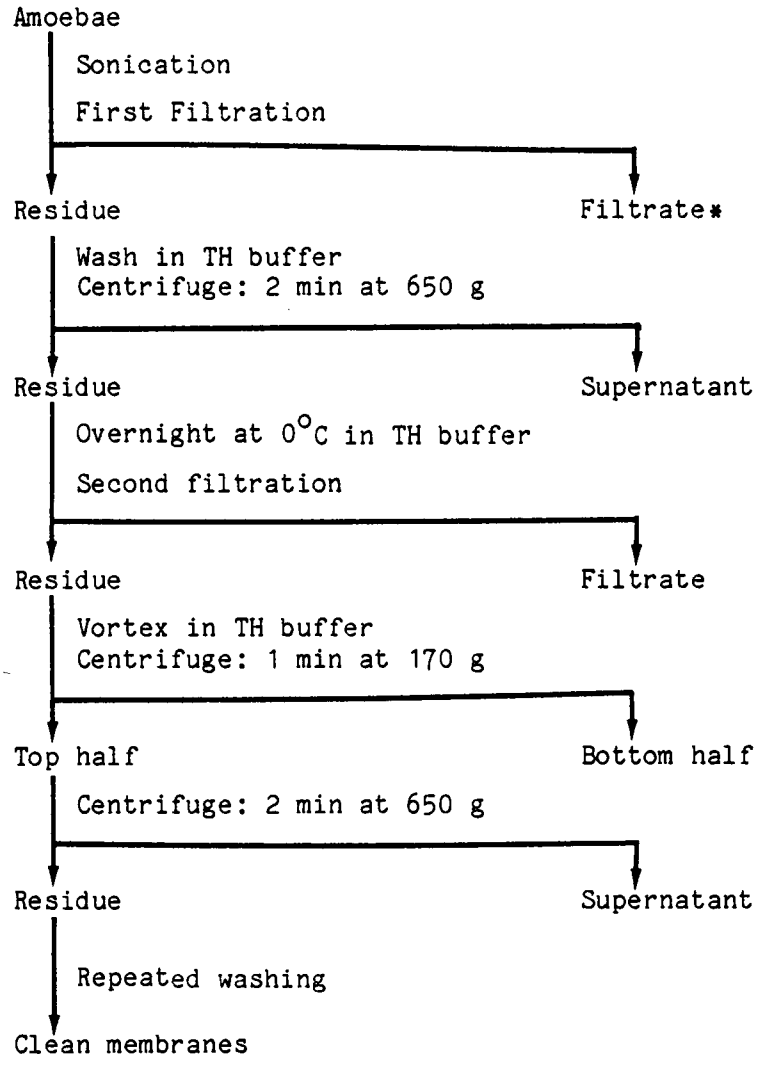

Figure 1. A procedure for isolating cell membranes

The filtrate* was saved to fractionate symbiotic vacuoles.

daily washing at room temperature and collected by centrifugation for 10 sec at 170 g. They were then washed twice in TH buffer and sedimented by centrifugation for 1 min at 170 g. The packed amoebae (8 ml starting volume chosen for convenience) were brought to 25 ml with 10 % sucrose, and kept for 10 min at 0 °C. Amoebae were then lysed by ultrasonication for 10 sec at 40 % maximum intensity using an 18-mm tip probe (standard tip) on a Fisher Dismembrator, Model 300. The amoeba lysate was filtered through 30-μm pore nylon screen, and the residue which contained large pieces of plasmalemma and nuclei was collected in 25 ml TH buffer. The suspension was sonicated as before and again filtered through the same screen. The screen was washed five times with 10 ml each of TH buffer. The collected residue was then centrifuged for 2 min at 650 g. The pellet was resuspended in 10 ml buffer, left overnight at 0 °C, and filtered through the same screen. After rinsing five times with TH buffer, the residue was collected in 4 ml buffer, vortexed for 10 sec, and centrifuged for 1 min at 170 g in a 12-ml conical centrifuge tube. The supernatant and the top half of the residue which contained most of the plasmalemma were collected, and the bottom half was recycled to remove contaminating nuclei. Membranes were recovered by centrifuging the pooled supernatant for 2 min at 650 g. The sediment was resuspended in 4 ml buffer, vortexed for 10 sec, and centrifuged for 1 min at 170 g. The supernatant and top half of the pellet were then collected. By repeating this process three times, it was possible to completely free the membrane fraction of contaminating nuclei.

PHAGOSOMES To prepare the phagosomes, 32.2-μm latex particles were used to get the maximum surface area. When the cells were incubated with particles diameter smaller than 20 μm, each phagosome contained several particles and the net phagosomal surface area decreased. When the latex particles were suspended in culture, they immediately attached on the cell surface, and settled at the bottom of the dish along with amoebae. Thus, phagocytosis was initiated immediately. Amoebae took up an average of 5, 9, and 12 particles per cell in 0.5, 1, and 1.5-hour incubation periods, respectively. When the cells were incubated for more than 1.5 hours, the number of induced phagosomes did not increase.

Phagosomes were isolated by the procedure outlined in figure 2. About 10^6 amoebae were starved for 2 days with daily washing in a mass-culture dish, and were mixed with 1.5 ml of packed polystyrene beads (32 μm in diameter) for phagocytosis. After a given period of time, 1 to 4 hours, free latex beads were removed by filtering the culture through 100-μm pore TET screen, and amoebae were collected from the screen top. After washing the cells in the Chalkley's, they were harvested in the TH buffer and sonicated in 10 % sucrose as in the isolation of plasmalemma. After filtering the lysate through 20-μm pore screen, the residue was collected in buffer and centrifuged for 5 min at 350 g. The residue, which contained all the phagosomes, large pieces of plasmalemma, and nuclei, was suspended in 4 volumes of 50 % sucrose and overlayed with 30 and 10 % sucrose. After centrifugation for 10 min at 4000 g, phagosomes

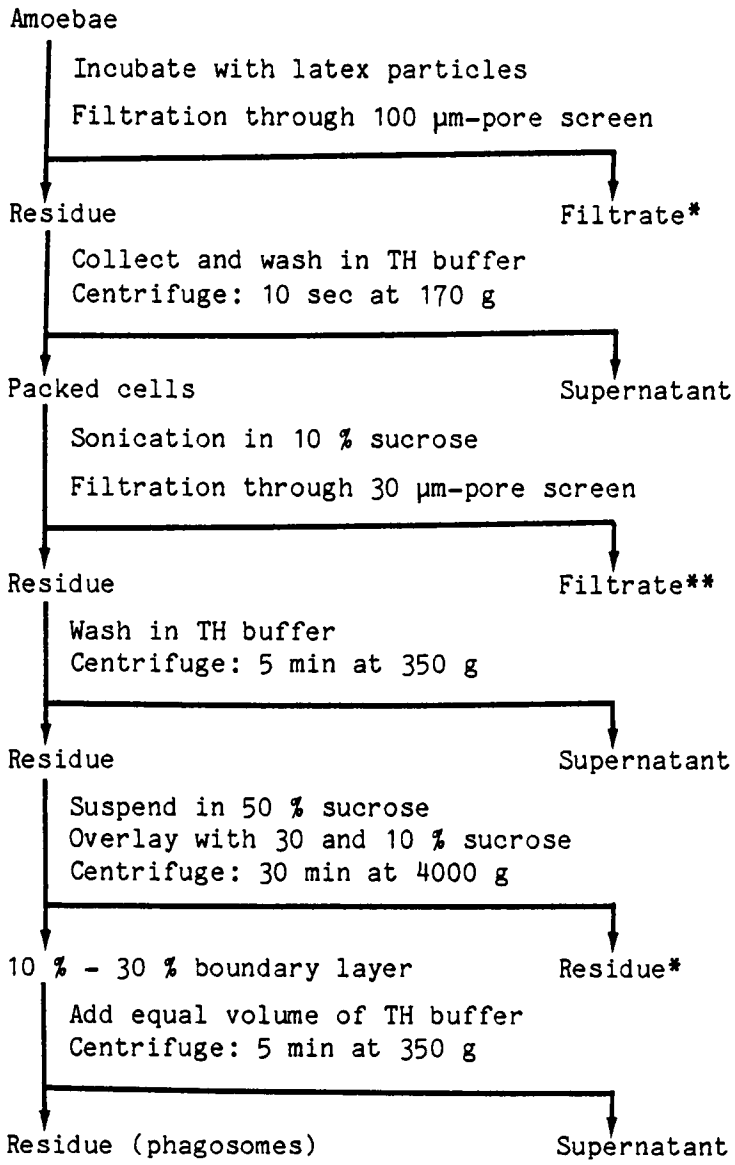

Figure 2. A procedure for isolating phagosomes

Latex beads were recycled from the filtrate*. The filtrate** and the bottom residue* was saved to fractionate symbiotic vacuoles and cell membranes, respectively.

were collected from the boundary between 30 and 10 % sucrose layers, washed in buffer, and collected by centrifugation for 5 min at 350 g.

For recovering the latex particles from the incubation medium, the particles were collected and sedimented in a beaker. Then the particles were suspended in 95 % sulfuric acid for a few hours, and washed extensively in distilled water. The surface of the particles was coated with poly-L-lysine (m.wt. 13,000) to give positive charges by incubating for 15 min in 1 % poly-L-lysine solution at 37 °C. The coated particles were pelleted by centrifugation and suspended in Chalkley's medium for inducing phagocytosis.

SYMBIOTIC VACUOLES Symbiotic vacuoles were very fragile, and burst immediately in Chalkley's medium. The homogenizing buffer system and sucrose concentration were empirically determined to preserve the integrity of the symbiotic vacuoles as much as possible in further processing of subcellular fractions. The routine procedure for isolating symbiotic vacuole is outlined in figure 3. The amoebae were starved for 3 days with daily washing in mass-culture dishes, harvested in ice-cold TH buffer and packed by centrifugation for 1 min at 170 g to measure the packed cell volume. The amoebae in 10 % sucrose were then lysed by ultrasonication as in the isolation of plasmalemma. The lysate was filtered successively through 30-μm and 20-μm pore screens. The filtrate contained symbiotic vacuoles and other contaminating cell organelles, but was free of nuclei and large cell membrane fragments. The filtrate was centrifuged for 5 min at 350 g. Then the supernatant

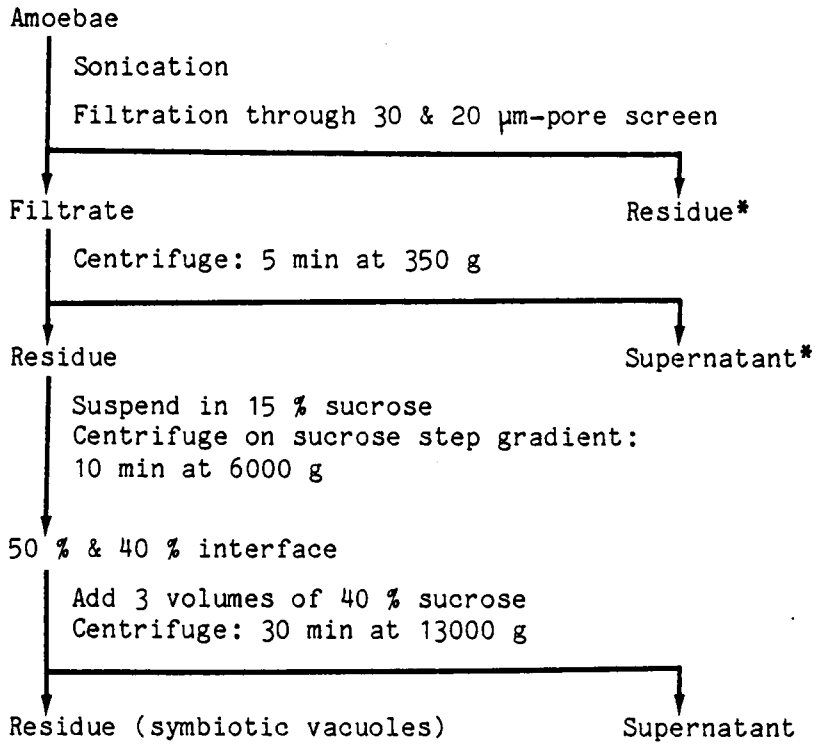

Figure 3. A procedure for isolating symbiotic vacuoles

The residue* and supernatant* were saved to fractionate cell membranes and bacteria, respectively.

containing most of the contaminating cell organelles and free bacteria, was removed. The pellet containing symbiotic vacuoles and crystals was suspended in 15 % sucrose, layered on top of a step gradient of 50, 40, and 30 % sucrose in four 30-ml tubes. Pure symbiotic vacuoles were collected from the 40 and 50 % boundary after centrifugation for 10 min at 6000 g. After adding 3 volumes of 40 % sucrose to the interface fraction, symbiotic vacuoles were pelleted by centrifugation for 30 min at 13,000 g, and finally resuspended in 10 % sucrose. For iodination, symbiotic vacuoles were taken from 40 and 50 % interface without further dilution. After iodination, symbiotic vacuoles were fractionated by the second step-gradient centrifugation as described above.

BACTERIA Pure bacteria were obtained either from the purified symbiotic vacuoles or from the supernatant of the filtrate in symbiotic vacuole preparation. The procedure is outlined in figure 4. Symbiotic vacuoles were ruptured by sonication for 15 sec at 30 % maximum intensity using a 10-mm tip probe (intermediate tip), vortexed in 10 % sucrose, and were layered on top of a step gradient of 40, 30, and 15 % sucrose. Pure bacteria were collected from 30 % layer after spinning for 10 min at 6000 g, and were washed twice in TH buffer by centrifugation for 20 min at 13,000 g.

When bacteria were collected from the supernatant of the filtrate, bacteria and other small cell organelles in the supernatant were pelleted by centrifugation for 15 min at 10,000 g. After freezing and thawing, the pellet was washed in TH buffer by centrifugation. Pure

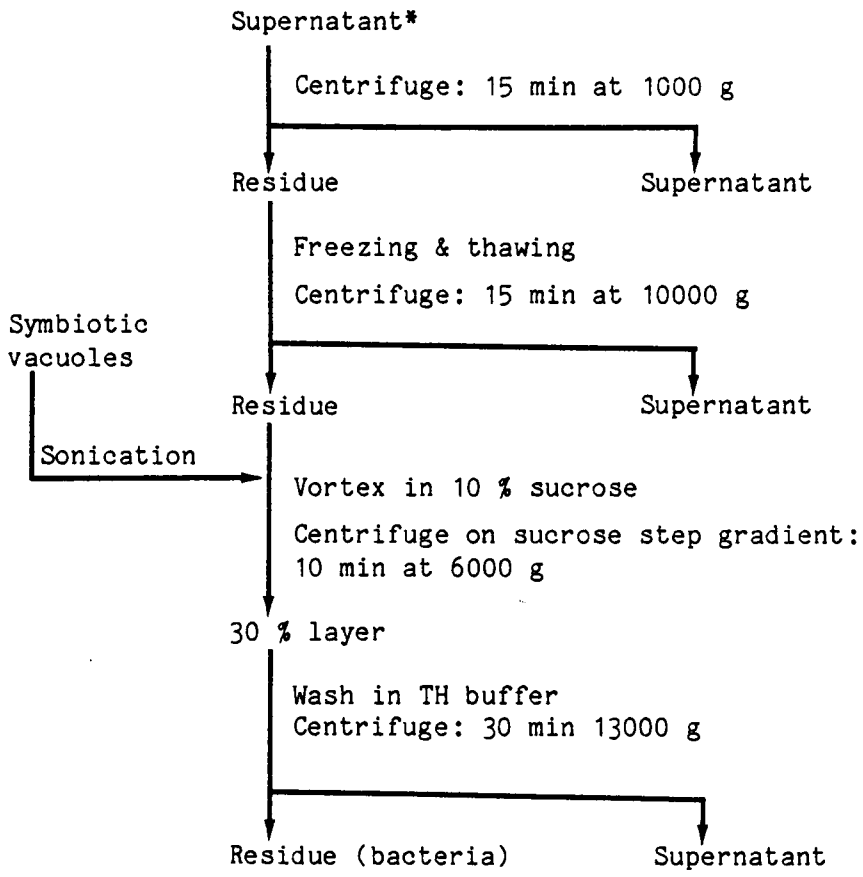

Figure 4. A procedure for isolating symbiotic bacteria

Supernatant* is from the procedure shown in figure 3.

bacteria were obtained by sucrose step-gradient centrifugation as described above.

4. Fluorescence Microscopy of Acridine Orange-Labeled Cells

The cells containing phagosomes labeled with polystyrene particles, were incubated for 10 min with 100 µg/ml acridine orange (AO) to label the secondary lysosomes (Hart & Young, 1975). The cells were washed thoroughly with fresh Chalkley's medium and kept in it for varying time periods. They were then mounted on 0.6 % agar slide (Jeon & Lorch, 1968). Fusion of the AO-labeled secondary lysosomes with latex particle-labeled phagosomes or with symbiotic vacuoles was studied under a fluorescence microscope (Leitz MPV2 microscope) using a mercury lamp. Pictures were taken with Kodak Tri-X pan film.

5. Electron Microscopy and Enzyme Cytochemistry

Transmission Electron Microscopy

Cells or subcellular fractions were fixed in cacodylate-buffered (0.1 M, pH 7.4) paraformaldehyde-glutaraldehyde (Karnovsky, 1965) for an hour at 4 °C and post fixed in 2 % buffered osmium tetroxide for an hour at room temperature. Fixed cells were dehydrated in a graded series of ethanol. Toluene was used as a transitional agent between the alcohol series and the embedding mixture. Finally, the samples were embedded in Epon 812 (Luft, 1961). Sections were cut on a Porter-Blum MT-2

ultramicrotome. Thick sections (0.3 μm thick) were cut and stained with 0.25 % Azure II on a glass slide (Jeon, 1965), and examined under light microscope. Thin sections with silver or gold color were mounted on 300-mesh copper grids and double stained with saturated uranyl acetate and lead citrate (Reynolds, 1963). The sections were examined and photographed in a Zeiss EM9S or Hitachi H-600 electron microscope.

Cytochemistry of Marker Enzymes

The aldehyde-fixed cells were incubated for each enzyme, post-fixed with osmium tetroxide for 40 min at 0 °C, and processed for electron microscopy. Stained or unstained thin sections were studied under the EM. For all enzyme incubations, lead nitrate and enzyme substrate solutions were prepared separately and filtered through 0.45-μm millipore filter to remove dust and other particles which might form foci for lead precipitants. The filtered nitrate solution was added slowly from a pipet whose tip was immersed in the solution containing the other components of the incubation medium, the resultant solution being constantly agitated.

Mg^{2+} Adenosine 5'-Triphosphatase Formaldehyde-fixed cells were incubated for an hour at 25 °C in 80 mM tris-maleate buffer (pH 7.4) containing 1.0 mM ATP, 10 mM magnesium sulfate, and 3.6 mM lead nitrate. The control group was fixed with glutaraldehyde which had been known as a potent inhibitor for extra adenosine 5'-triphosphatase (ATPase) (Hayat, 1973).

5'-Nucleotidase Cells were fixed for 10 min with 1 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 0 °C. The fixed and buffer-washed cells were then incubated for an hour at 25 °C in Widnell's medium (Widnell, 1972) which contained 1 mM 5'-AMP, 1 mM lead nitrate, and 1 mM magnecium nitrate in 0.1 M tris-maleate buffer, pH 7.5. For the control group, sodium floride was added to a concentration of 10 mM to inhibit the enzyme activity (Farquhar et al., 1974).

Glucose 6-Phosphatase Cells were fixed for 30 min at 0 °C in tris-maleate buffered (0.1 M, pH 6.7) glutaraldehyde (3 %). Then the fixed cells were washed with buffer and were incubated for an hour at 25 °C in a medium containing 2 mM glucose 6-phosphate (G6P) and 3.6 mM lead nitrate in 0.1 M tris-maleate buffer, pH 6.7.

Thiamine pyrophosphatase Cells were fixed for an hour at 0 °C in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After washing with buffer, the cells were incubated for an hour at 25 °C in a medium containing 1 mM TPP, 3.6 mM lead nitrate, and 0.5 mM $MnCl_2$ in 0.1 M tris-maleate buffer, pH 7.4 (Wise & Flickinger, 1970a).

Acid phosphatase Paraformaldehyde- and glutaraldehyde-fixed cells were incubated for an hour at 25 °C in medium containing 10 mM sodium β-glycerophosphate and 3.6 mM lead nitrate in 0.05 M acetate buffer, pH 5.0 (Faquhar et al., 1974).

6. Assay of Membrane Marker Enzymes

Protein assay The amount of proteins in each sample was measured by colorimetry with Gilford 240 Spectrophotometer following Lowry et al., (1951). Samples were solubilized in 1 % SDS, supernatants were then taken for assay after centrifugation for 30 min at 1400 g. The standard protein, bovine serum albumin, was also prepared in 1 % SDS.

Assay of inorganic phosphate After enzyme incubation, the amount of released free inorganic phosphate was measured according to the Sigma bulletin method (No.670, 1974), which was a modification of Fisk & Subbarow (1925). KH_2PO_4 was used as the standard.

The original formulas of all enzyme assays, except assays for acid phosphatase, were modified to a final incubation volume of 1 ml. After incubation for a certain time period at 37 °C, the reaction was stopped by adding trichloroacetic acid (TCA). After centrifugation for 10 min at 1400 g, 0.8 ml of the supernatant was taken for assay of inorganic phosphate.

Assay of p-nitrophenol Specific enzyme activity of acid phosphatase and alkaline phosphodiesterase was assayed by measuring the amount of released p-nitrophenol from the substrate according to the Sigma bulletin method (No.104, 1974). Disodium p-nitrophenol was used as the standard.

Acid phosphatase (E.C.3.1.3.2) Acid phosphatase (AcPase) activity was assayed according to the Sigma bulletin method (No.104, 1974) in a final incubation volume of 240 µl. The reaction was initiated by adding 40 µl of an enzyme sample (0.3-1.0 mg protein/ml) to incubation medium containing 7.5 mM p-nitrophenolphosphate in 45 mM citrate-HCl buffer (pH 4.8). After incubation for 30 min at 37 °C, the reaction was stopped and color was developed by adding 1 ml of 0.1 N NaOH. Potassium floride was used as an inhibitor of this enzyme at a concentration of 50 mM.

Alkaline phosphodiesterase I (E.C.3.1.4.1) The activity of alkaline phosphodiesterase I was assayed according to Beaufay et al., (1974). The reaction was initiated by adding 100 µl of an enzyme sample (about 100 µg protein) to 900 µl of incubation medium containing 2 mM zinc acetate, and 1.5 mM p-nitrophenyl N-acetyl-D glucosaminide in 0.1 M glycine-NaOH buffer, pH 9.6. After incubation for 15 min at 25 °C, the reaction was terminated and color was developed by adding 400 µl of 1 N NaOH. Isobutylmethyl xanthine was used as an inhibitor of the enzyme at a concentration of 1 mM.

5'-Nucleotidase A modified method of Dixon and Purdon (1954) was used in which 5'-nucleotidase (5'-ribonucleoside phosphohydrolase, E.C.3.1.3.5) (5'-NTPase) activity was the difference between total phosphatase activity with AMP as substrate and acid phosphatase activity with sodium β-glycerophosphate as a substituted substrate (Sigma Bulletin No.675, 1978). Assay was performed in duplicates in a total

volume of 1 ml using about 100 µg protein per tube. Incubation was done for 2 hours at 37 °C in a medium containing 5 mM $MgCl_2$, 2.5 mM AMP or 2.5 mM sodium β-glycerophosphate in 0.1 M tris-HCl, pH 7.5.

(Na⁺-K⁺)- and Mg^{2+}-ATPase Assay was performed in triplicates in a total volume of 1 ml using about 100 µg protein per tube. Protein samples were added in a volume of 100 µl to series of tubes containing as follows; (1) 20 mM KCl, 130 mM NaCl, 3 mM $MgCl_2$ and 3 mM ATP in 10 mM tris-maleate, pH 7.5; (2) 3 mM $MgCl_2$ and 3 mM ATP in 80 mM tris maleate; and (3) 1 mM EDTA, and 3 mM ATP in 80 mM tris-maleate. (Na^+-K^+)ATPase activity is the difference between the phosphatase activity of (1) and (2), and Mg^{2+}ATPase (E.C.3.6.1.3) activity is the difference between that of (2) and (3). In the last series, 1 mM EDTA was added to inhibit Mg^{2+}ATPase activity. The reaction was initiated by adding enzyme samples, then terminated by adding 100 µl of 50 % TCA after incubation for 10 min at 37 °C. Ouabain was also used as an inhibitor of (Na^+-K^+)-ATPase at a concentration up to 20 mM.

Glucose 6-Phosphatase G6Pase activity was assayed according to Beaufay et al. (1974). Incubation was done for 30 min at 37 °C in a medium containing 10 mM G6P, 1 mM EDTA in 20 mM maleate buffer pH 6.5.

7. Iodination of Subcellular Fractions

The outer surface of intact cells and the cytoplasmic surface of subcellular fractions were ^{125}I-iodinated in an ice-box by

lactoperoxidase (LPO) (Phillips & Morrison, 1971). Two ml of the intact cells were harvested and washed with cold Chalkley's medium and suspended in 4 ml of 0.02 M tris-HCl in the Chalkley's (pH 7.4) in a 25-ml beaker for iodination. Subcellular fractions were also suspended in an appropriately buffered sucrose solution, phagosomes and free bacteria in 10 % sucrose and symbiotic vacuoles were taken from 40 - 50 % boundary as described previously. Then, 50 µl of 2 mg protein/ml LPO and 200 µC of carrier-free NaI^{125} were added to each of the samples and mixed by gentle shaking. Iodination was then initiated by adding 10 µl of freshly prepared 1 mM hydrogen peroxide. After adding hydrogen peroxide in 20 successive installments at 30-sec intervals, the reaction was stopped by adding 5 µl of 0.2 M sodium metabisulfite prepared just before use. Then 50 µl of 50 mM KI were added. The incubation mixture was diluted to 10 ml with TH buffer.

The iodinated cells were washed 3 times in ice-cold Chalkley's medium by centrifugation for 10 sec at 170 g, and dialyzed against TH buffer containing 5 mM EDTA overnight with three changes of buffer. The cells were broken by sonication for 10 sec at 25 % maximum intensity using a 10-mm tip probe (intermediate tip). The homogenate was centrifuged for 10 min at 1400 g. After removing the supernatant, the pellet which contained cell membranes, nuclei, and other heavy organelles was washed again in TH buffer by centrifugation. The washed pellet was used to examine the iodinated protein pattern of the outer cell surface.

The suspension of iodinated symbiotic vacuoles was diluted by adding an equal volume of 30 % sucrose, layered on top of 40 and 50 % sucrose step gradient, and centrifuged to get iodinated pure symbiotic vacuoles from 40 and 50 % interface. Then the symbiotic vacuoles were further washed by centrifugation and dialyzed as above.

The iodinated phagosomes and bacteria were also washed 3 times in TH buffer by centrifugation for 5 min at 700 g, and for 30 min at 10,000 g, respectively. Then these samples were further dialyzed.

Radioactivity of the iodinated samples was measured by disc method (Hubbard & Cohn, 1975). Air-dried discs placed in tubes were counted in a gamma counter (Beckman Gamma 4000) whose counting efficiency was 0.7.

8. SDS Polyacrylamide Gel Electrophoresis and Gel Radioautography

SDS Polyacrylamide Gel Electrophoresis (PAGE) Laemmli's gel and buffer system (Laemmli, 1970) were employed for SDS PAGE. The separating gel was 8 % gel in 0.375 M tris-HCl pH 8.8, containing 0.1 % SDS. The stacking gel was 3.3 % gel in 0.125 M tris-HCl pH 6.8 containing 0.1 % SDS. The gel was cast in a slab with 1.5 mm in thickness. Samples containing 60-120 µg protein in 50 µl were applied after boiling for 1.5 min in a solution containing 1 % SDS, 5 % mercaptoethanol, 0.002 % bromophenol blue, 2.5 % sucrose and 0.125 M tris-HCl pH 6.8 with or without 0.002 M phenylmethylsulfonylfluoride. After filling the upper and the lower tanks of the apparatus with

0.025 M tris-glycine buffer pH 8.3 containing 0.1 % SDS, electrophoresis was run for 9 to 11 hours at 150 V, 0.1 discharge capacitance, 400 pulses/min using Ortec 4100 pulsed constant power supply (Oak Ridge, TN) until the tracking dye (bromophenolblue) migrated to a point 0.5 cm from the bottom of the gel. Then the gel was fixed and stained with brilliant coommassie blue (BCB) or periodic acid Schiff (PAS) reagent following the method of Fairbanks et al., (1971).

Gel-radioautography When ^{125}I-iodinated samples were electrophoresed, gels were extensively destained and pretreated for an hour in a solution containing 1 % glycerol and 10 % acetic acid. The gels were then dried on 3MM Whatman chromatography paper for 4 hours in a slab gel dryer (Hoefer Scientific Instruments, San Francisco, CA.). Radioautograms were made from the dried gel after various time periods of exposure (1-7 days) in a deep-freezer (-70 °C) using Kodak X-Omat R film.

CHAPTER III

RESULTS

1. Lysosomal Fusion

A. Acridine-Orange Fluorescence Microscopy

The amoebae that had been incubated for an hour and half with latex beads as described in Materials and Methods took in about 12 latex beads on the average. Fusion of the secondary lysosomes with thus-formed phagosomes was shown by acridine orange fluorescence microscopy (Fig. 5). Following incubation for 20 min in 100 μg/ml of acridine orange, the cells were highly fluorescent. Immediately after incubation with acridine orange, the cells were full of fluorescent pinocytic vesicles. The fusion of the secondary lysosomes with the phagosomes or other cell organelles was best shown after overnight. Accumulation of acridine orange in phagosomes containing latex beads was distinct (Fig, 5B), but fusion of lysosomes with symbiotic vacuoles was not detectable. On any slight damage to the cell during sampling for microscopy, the fluorescence diffused all over the cell. The diffused acridine orange was intensly fluorescent in the cell nucleus and symbiotic bacteria. A slide with supporting coverslips or an agar slide was suitable for the observation of AO-labeled cells. The agar slide had an advantage for the cells to spread without any physical or chemical demage. When the cells were fixed with acetic acid, ethanol or 4 % formaldehyde, there was much background fluorescence due to autofluorescence of the

Figure 5. Fusion between acridine orange-labeled secondary lysosomes and phagosomes

A. Phase-contrast micrograph of an xD amoeba containing latex beads in phagosomes (arrows).

B. Fluorescence micrograph of the same amoeba as in A with fluorescence in phagosomes by fusion of AO-labeled secondary lysosomes.

The bars represent 50 μm (x530).

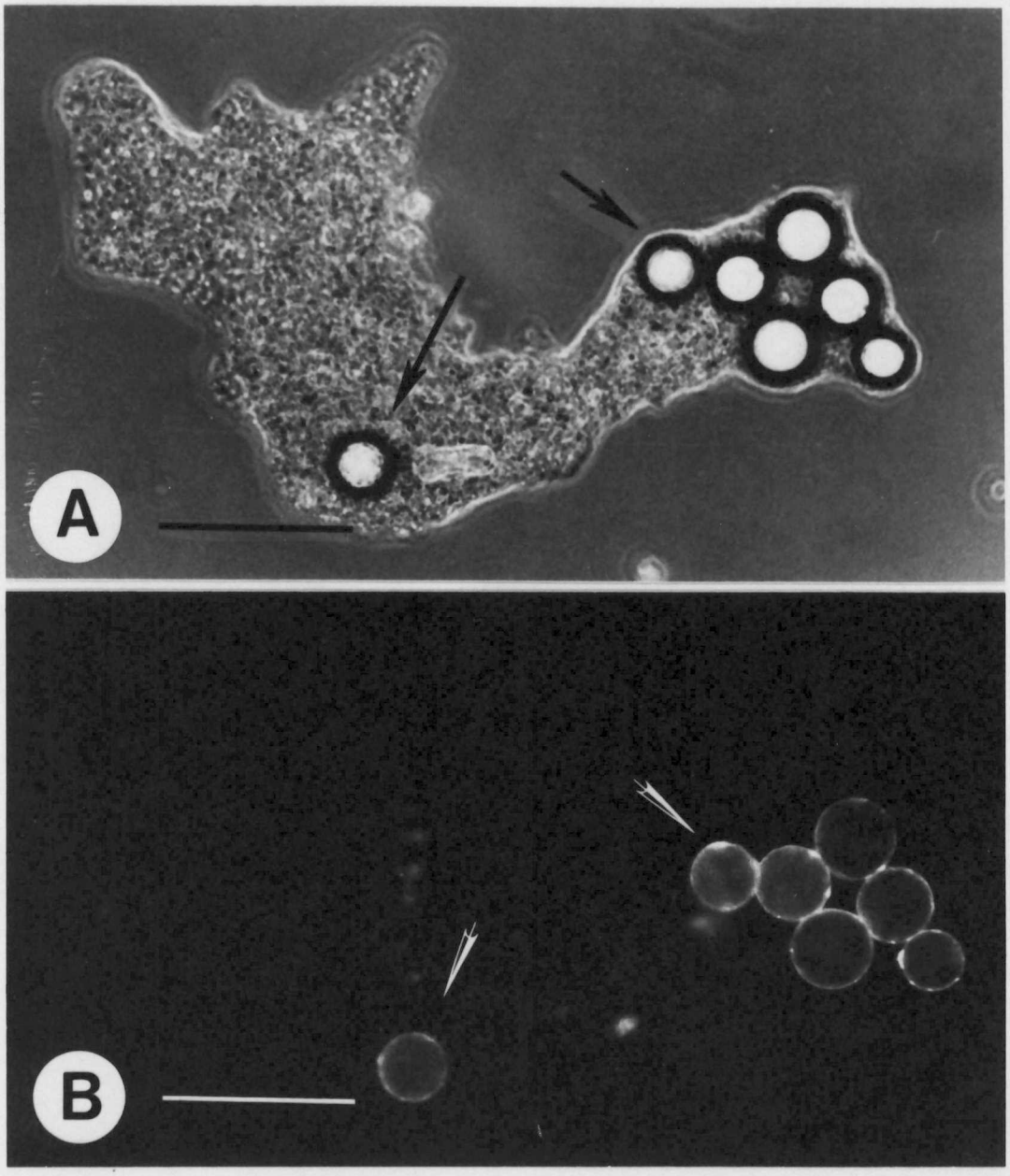

Figure 5

fixative, or due to the diffused fluorescence that had been caused by damage on the membranes during chemical fixation.

B. Electron Microscopic Study of Lysosomal Fusion

The cells which had been incubated with ferritin for pinocytosis were fixed after overnight in the Chalkley's medium. Thin sections of embedded samples were examined in an electron microscope with or without staining. The electron-dense deposit of ferritin was shown in the pinocytic vesicles and around latex beads within phagosomes (Fig. 6). Ferritin was known to be taken in by pinocytosis (Chapman-Andresen & Christensen, 1970). The pinocytic vesicles containing ferritin fused with lysosomes to form secondary lysosomes. The presence of ferritin in the phagosomes was a direct evidence for fusion of lysosomes. On the other hand normal symbiotic vacuoles did not show any dense deposits. The above two experiments showed that there was a clear difference between symbiotic vacuoles and phagosomes in fusing with lysosomes. The absence of acid phosphatase activity in symbiotic vacuoles (Jeon & Jeon, 1976; Chap. III,3) was proven to be due to the absence of lysosomal fusion rather than an inhibition of enzyme activity in symbiotic vacuoles.

Figure 6. Electron micrograph showing lysosomal fusion in an xD amoeba

Electron-dense deposits of ferritin are shown around the latex beads (LB) of the phagosome. But no deposit is shown in the symbiotic vacuole (SV).

The bar represents 1 μm (x10,500).

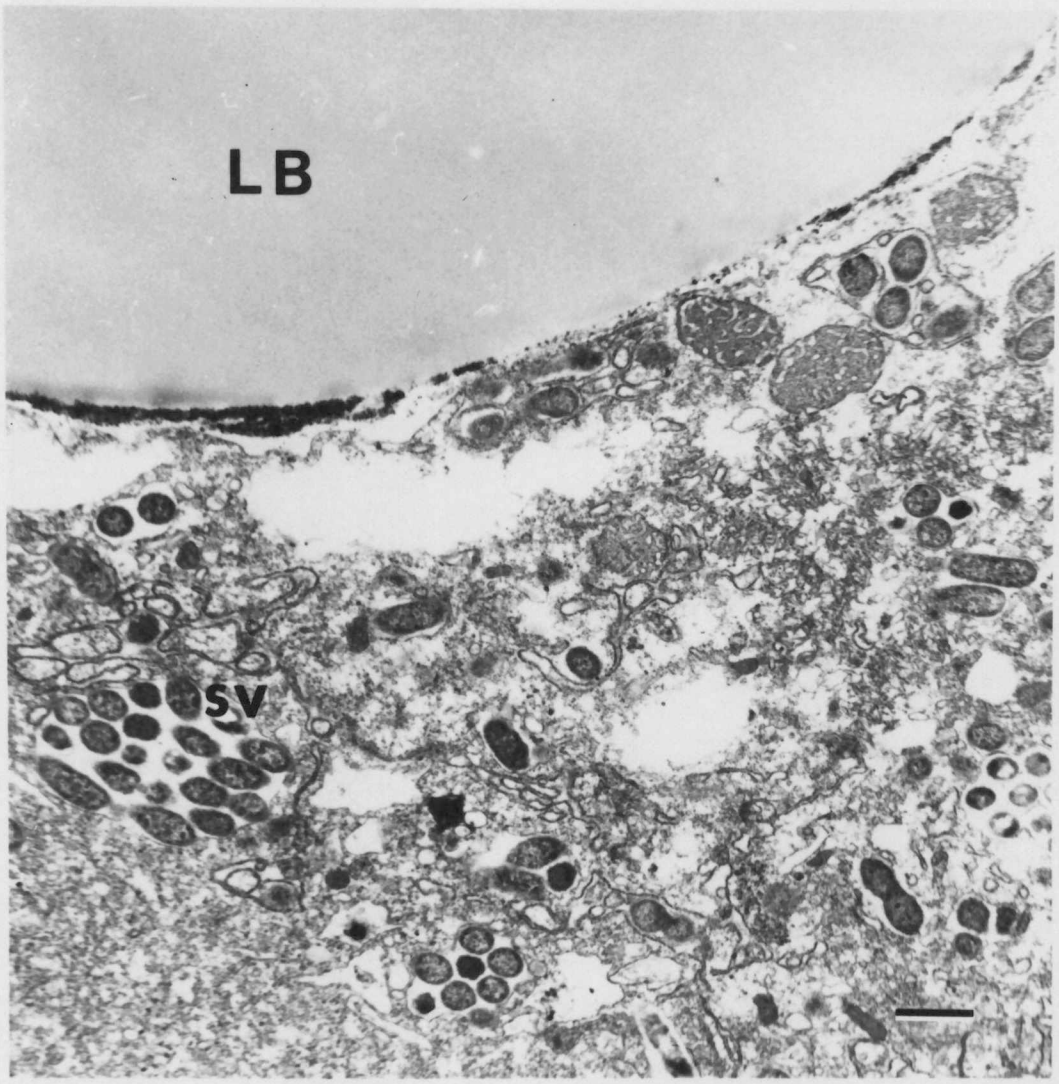

Figure 6

2. Phagocytosis and Changes in Membrane Morphology

Changes in morphology of the plasmalemma were followed by electron microscopy of amoebae that had been incubated with latex beads to induce phagocytosis. The cells appeared to attach on the surface of latex beads by fringes (Fig. 7A). The latex beads appeared to be taken in by simple invagination of the plasmalemma. There were no recognizable receptor sites or capping phenomena associated with the endocytosis of latex beads in amoebae. The surface of the invaginating plasmalemma maintained its fringes and the underlying plasma membrane (Fig. 7B). After 2 hours of endocytosis, the morphology of phagosomal membranes was different from that of the plasmalemma (Fig. 7C). The glycocalyx of the plasmalemma was almost completely lost. The latex beads were surrounded by plasma membranes only. The endocytosed plasmlemma appeared to lose its glycocalyx by cellular digestion. This observation was important in interpreting the results of SDS PAGE of phagosomes (Chap. III,6).

3. Cytochemistry of Marker Enzymes

In order to study changes in the functional aspect of the vacuole membranes, aldehyde-fixed cells were incubated for assay of enzyme activities of membrane markers. Among the various marker enzymes studied, acid phosphatase and glucose 6-phosphatase were detected by cytochemistry. Thin sections of embedded samples were examined in the

Figure 7. Morphology of the glycocalyx of the cell surface following endocytosis and digestion

A. The cell is attached to the surface of latex bead (LB) by glycocalyx (arrow). The unit membrane is indicated by an arrow head. (85,600).

B. An endocytosing amoeba. The glycocalyx (arrow) appears to be intact. (x100,000).

C. A phagosome 2 hours after ingestion. The unit membrane is intact, but the glycocalyx is mostly lost. (x115,000)

Bars represent 0.1 μm.

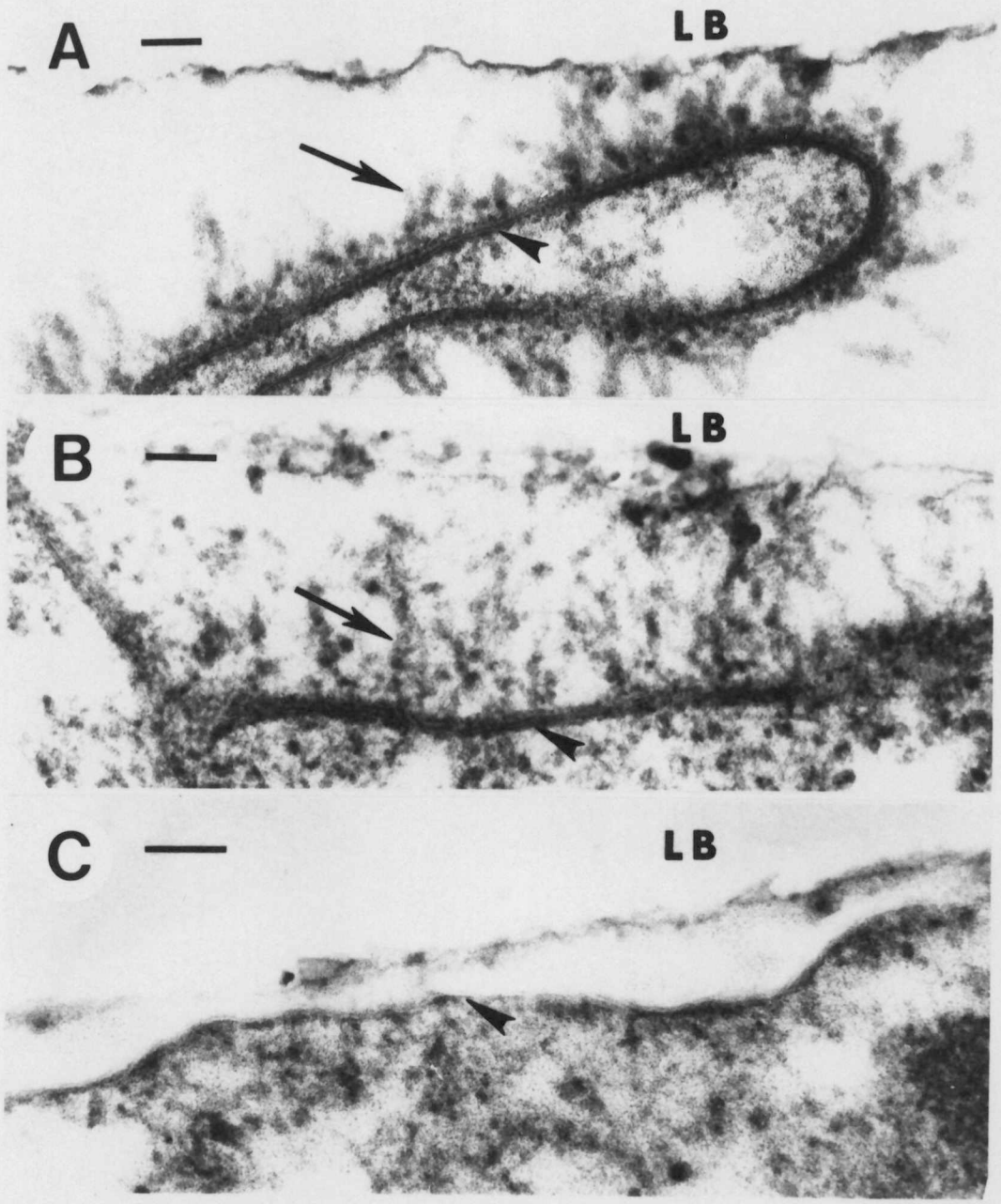

Figure 7

electron microscope after staining with uranyl acetate and lead citrate or with uranyl acetate only.

ACID PHOSPHATASE Electron-dense reaction products of acid phosphatase were found around latex beads in the phagosomes (Fig. 8). Such an electron-dense deposit of reaction products was not detectable in symbiotic vacuoles. This observation agreed with the previous study by Jeon and Jeon (1976).

GLUCOSE 6-PHOSPHATASE The electron-dense deposit of reaction products was present over the endoplasmic reticulum (Fig. 9). The activity was not shown either on cell membranes or symbiotic vacuole membranes. In this cell system, none of the well-known plasma membrane markers, such as, (Na^+-K^+)-ATPase, and 5'-NTPase, was detectable by enzyme cytochemistry.

4. Purity and Morphology of Subcellular Fractions

The dense crystals that usually contaminated subcellular fractions of large cell organelles were completely filtered through nylon screen. Without filtration, crystals were trapped by membrane, and were difficult to remove by centrifugation. Once the cell membranes and nuclei were separated from the whole homogenate, the crystals were readily pelleted at the bottom of the tube by centrifugation on a sucrose step-gradient. Thus, symbiotic vacuoles were completely free of contaminating crystals. Normal morphology of the cellular organelles

Figure 8. Enzyme cytochemistry of acid phosphatase in the xD amoeba

A. Electron-dense deposits of reaction products are shown around latex bead (LB) in the phagosome. Sections were stained with uranyl acetate and lead citrate. (x5,400)

B. The deposits of reaction products are not seen in the symbiotic vacuoles. Sections were stained with uranyl acetate only. (x21,600).

Bars represent 1 μm.

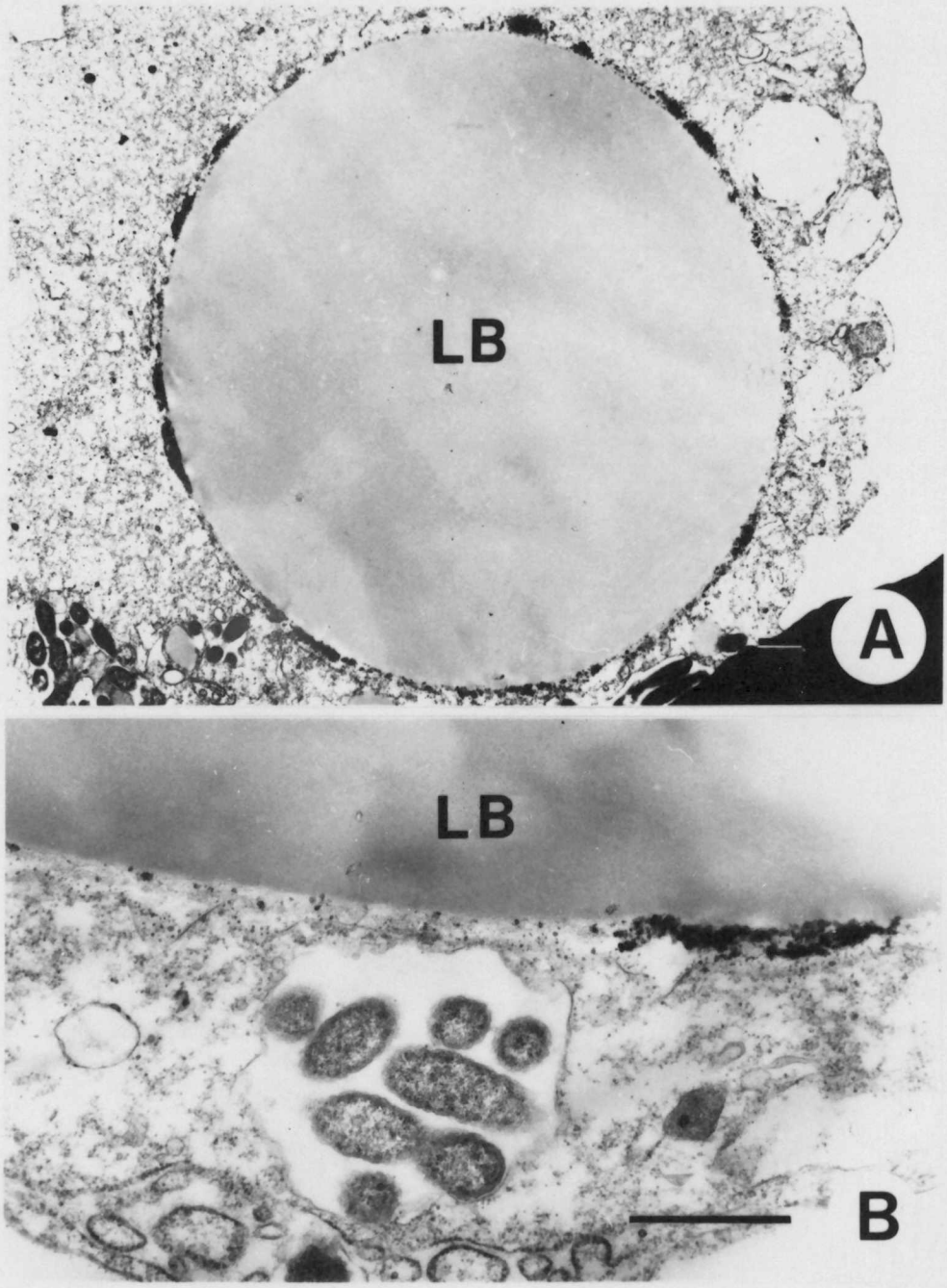

Figure 8

Figure 9. Enzyme cytochemistry of glucose 6-phosphatase in the xD amoeba

A. Fixed amoebae were incubated without the enzyme substrate. Thin section was stained with uranyl acetate only. The arrow indicates endoplasmic reticulum. (x71,200)

B. When amoebae were incubated with the enzyme substrate, electron-dense deposits of reaction products appeared over the endoplasmic reticulum (arrow). (x100,800)

Bars represent 0.2 μm.

Figure 9

before isolation is shown in figure 10.

PLASMALEMMA The purified plasmalemma was white in color after repeated washing by centrifugation (Chap. II,3). Under the phase-contrast microscope, the purified plasmalemmas were in rolled fragments (Fig. 11A). The fragmented membranes did not undergo any shape changes when the ionic strength of the buffer solution was changed. Thin sections of embedded samples did not show any contaminating organelles. Most of the membrane fragments were in rolled sheets (Fig. 12A). At a high magnification, the fringes and the unit membrane structure were still discernable. The membrane yield by this procedure was 2.85 mg of freeze-dried membrane/ml of packed wet cells.

PHAGOSOMES The occasional contaminants in the purified phagosomes were molds rather than other cellular organelles of amoebae. Free latex beads attached to molds and were fractionated with phagosomes. Cleaning amoebae before incubating with latex beads was important in obtaining a pure phagosome preparation. The minor contaminants at the final step of purification could be further removed by vortexing and running another sucrose step-gradient centrifugation. When the purified phagosomes were examined under a dark-field microscope or electron microscope at a low magnification, the bound phagosomal membranes were not readily visible (Fig. 13A). Purified phagosomes did not attach each other. When there were contaminating molds, the phagosomes and free latex beads clumped together. At a high magnification , the phagosomal membrane could be

Figure 10. Morphology of cellular organelles before isolation

A. Light micrograph of a thick resin section of an amoeba containing latex beads (LB) in phagosome. The bar represents 10 μm. (x1,600).

B. A typical plasmalemma (P) of an amoeba with the glycocalyx on the outer surface. The bar represents 1 μm (x16,400).

C. Symbiotic vacuoles (SV) of an amoeba. The vacuoles are bounded by a unit membrane only. The bar represents 5 μm (x3,700).

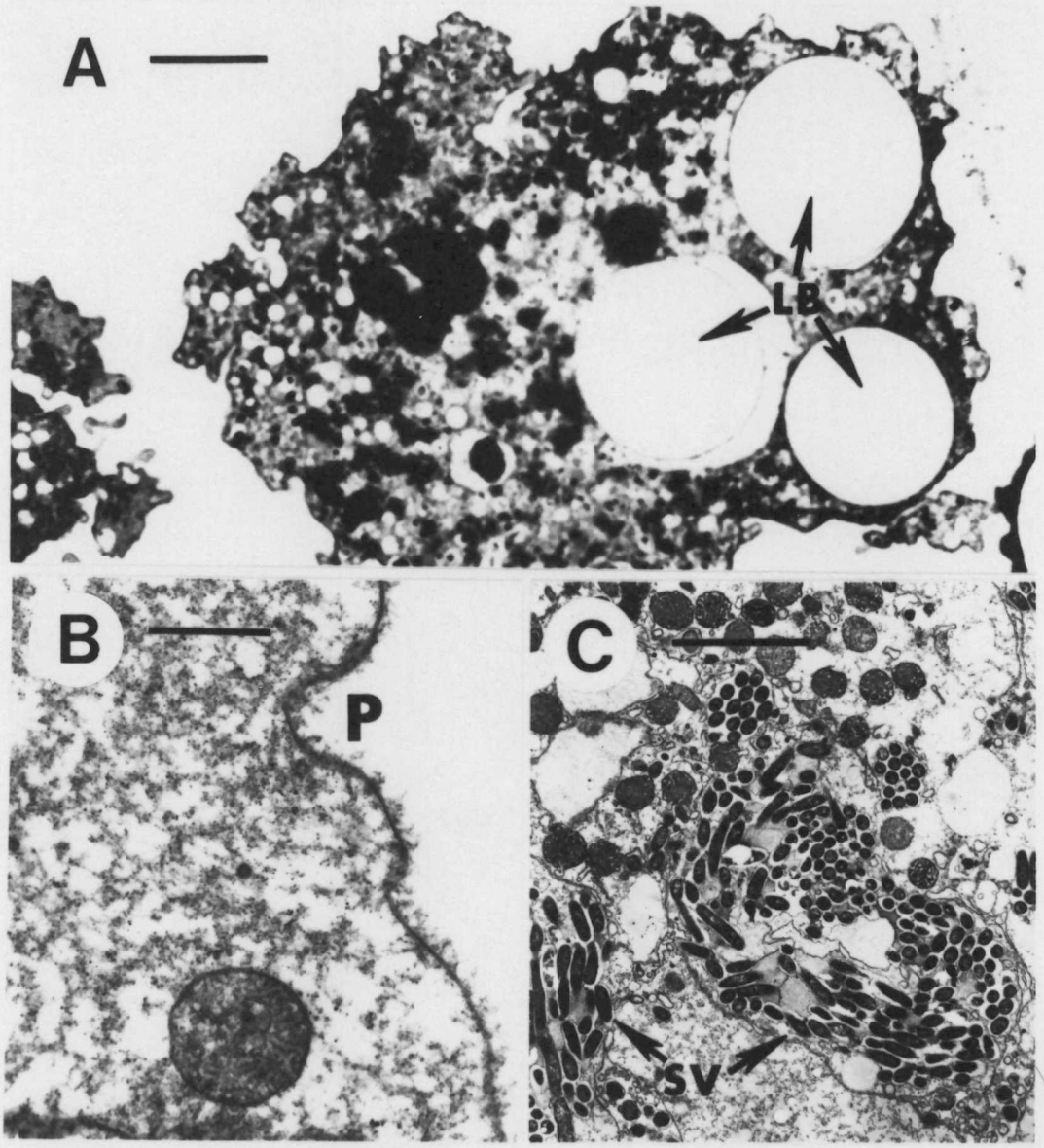

Figure 10

Figure 11. Purified plasmalemma at a low magnification

A. Isolated plasmalemma under the phase-contrast microscope. The bar represents 10 μm (x850).

B. Electron micrograph of isolated plasmalemma at a low magnification. The bar represents 5 μm (4,500).

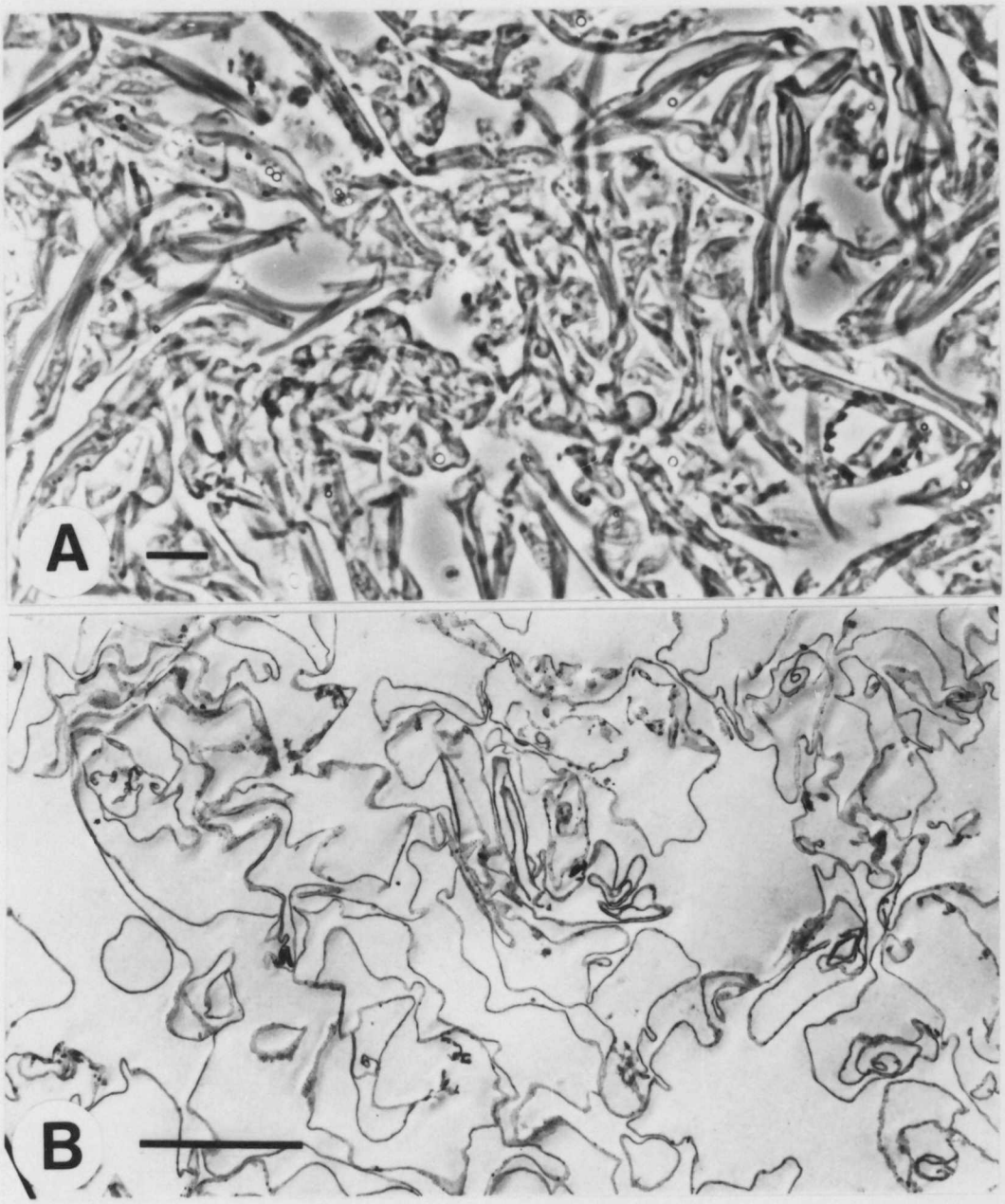

Figure 11

Figure 12. Ultrastructure of purified plasmalemma

A. The purified plasmalemma appeared as curled sheets. The bar represents 1 μm (x24,900).

B. Presence of the unit membrane (arrow head) and the glycocalyx (arrow)in the isolated plasmalemma. The bar represents 0.1 μm (x167,000).

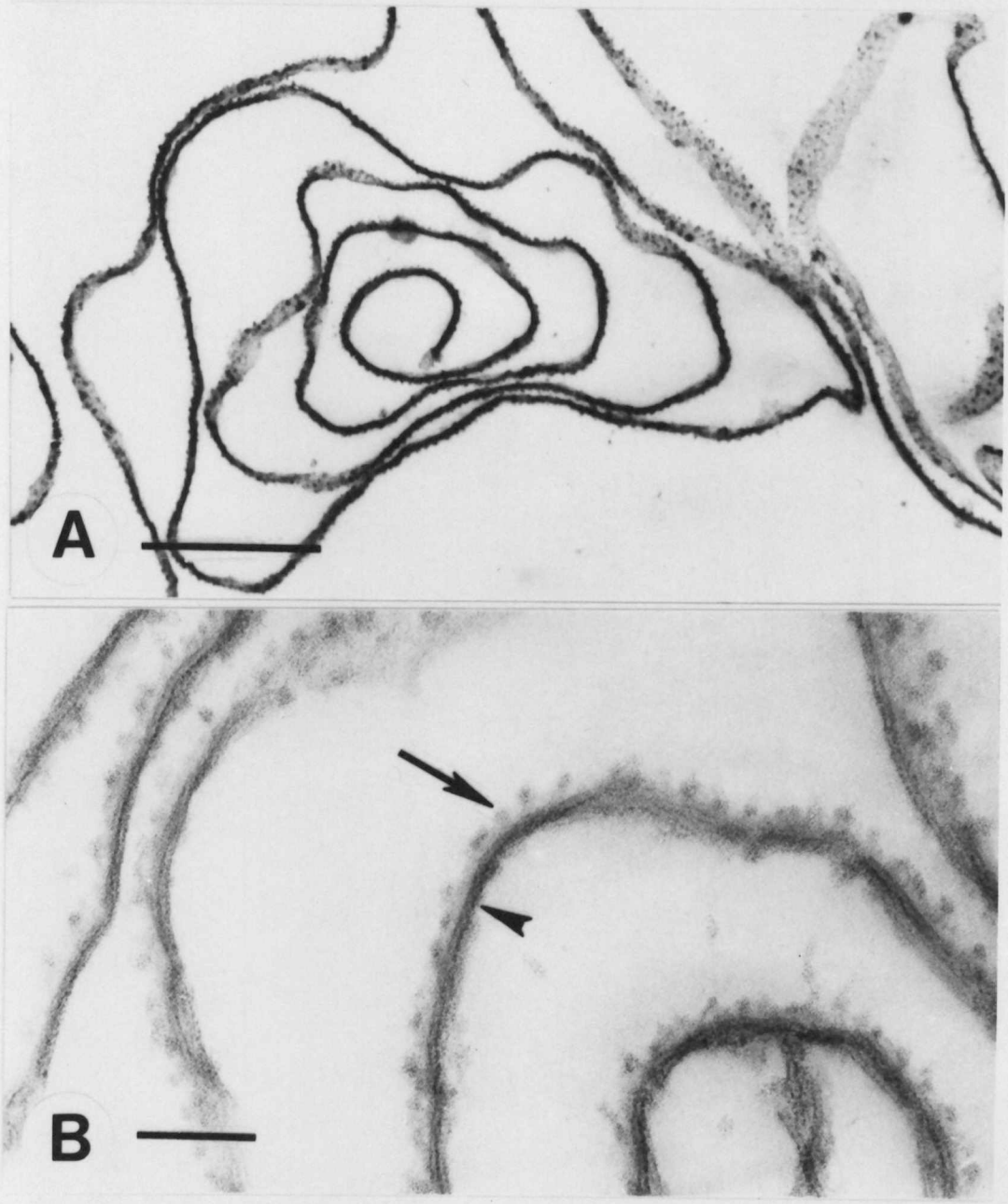

Figure 12

Figure 13. Electron micrographs of purified phagosomes containing latex beads

A. Purified phagosomes (Ph) at a low magnification. The bar represents 5 μm (x2,600).

B. Intact phagosomal membrane (arrow) is shown around the latex bead (LB) of a purified phagosome. The bar represents 0.1 μm (x82,700).

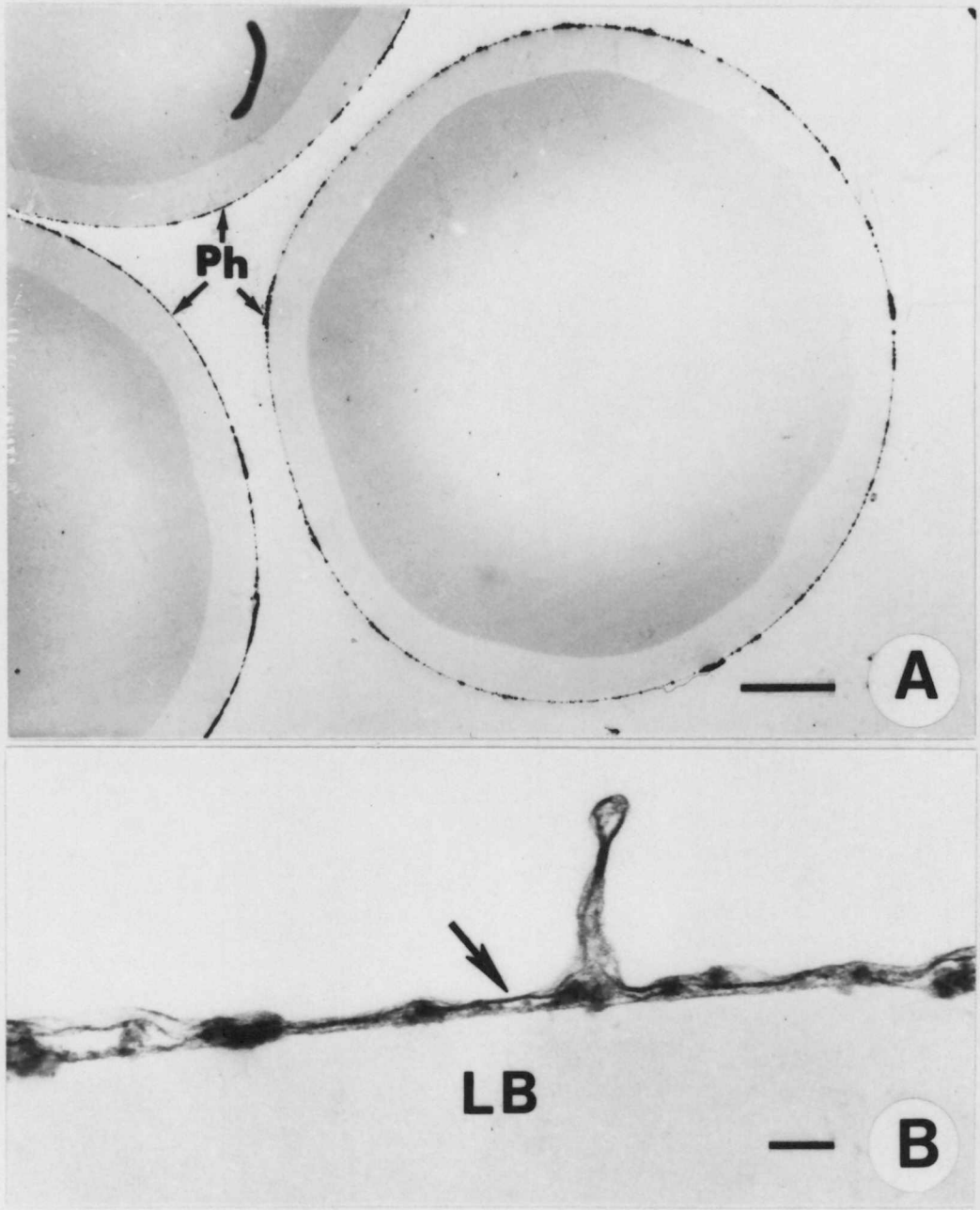

Figure 13

seen around the latex beads by electron microscopy (Fig. 13B).

SYMBIOTIC VACUOLES As symbiotic vacuoles were fractionated in a concentrated sucrose solution, their membranes broke easily on returning to a hypotonic solution. The purified symbiotic vacuoles appeared intact when examined under the phase-contrast microscope immediately after isolation. After fixation in aldehyde and washing in buffer, many of the vacuole membranes ruptured. But the overall configuration of the vacuole was maintained due to fixation. An osmotic effect on symbiotic vacuoles and bacteria during the sample preparation for electron microscopy could be seen by comparing the shape of bacteria in figure 10C and figure 14B. In figure 14B, the shape of bacteria was disfigured. Some of the bound membranes of symbiotic vacuoles were intact, but others were fragmented (Fig. 14C). If there was any damage on symbiotic vacuoles before fixation, bacteria readily burst out of the vacuoles. The yield of symbiotic vacuoles was about 15 % as measured in terms of the number of bacteria. There was a minor contamination by small fragments of the plasmalemma. These contaminants were not visible in the thick section of embedded samples when examined under the light microscope or at a low magnification in the electron microscope.

BACTERIA The bacterial preparation obtained either from symbiotic vacuoles or from the supernatant of amoeba homogenates as described in Chapter II, appeared clean under the phase-contrast microscope. The most pure symbiotic bacteria with less damage could be prepared from

Figure 14. Morphology of purified symbiotic vacuoles

A. Light micrograph of a thick resin section of symbiotic vacuoles. The bar represents 10 μm (x1,570).

B. Electron micrograph of purified symbiotic vacuoles. The bar represents 1 μm (x5,940).

C. A symbiotic vacuole with its boundary membrane (arrow). The bar represents 0.1 μm (x81,200).

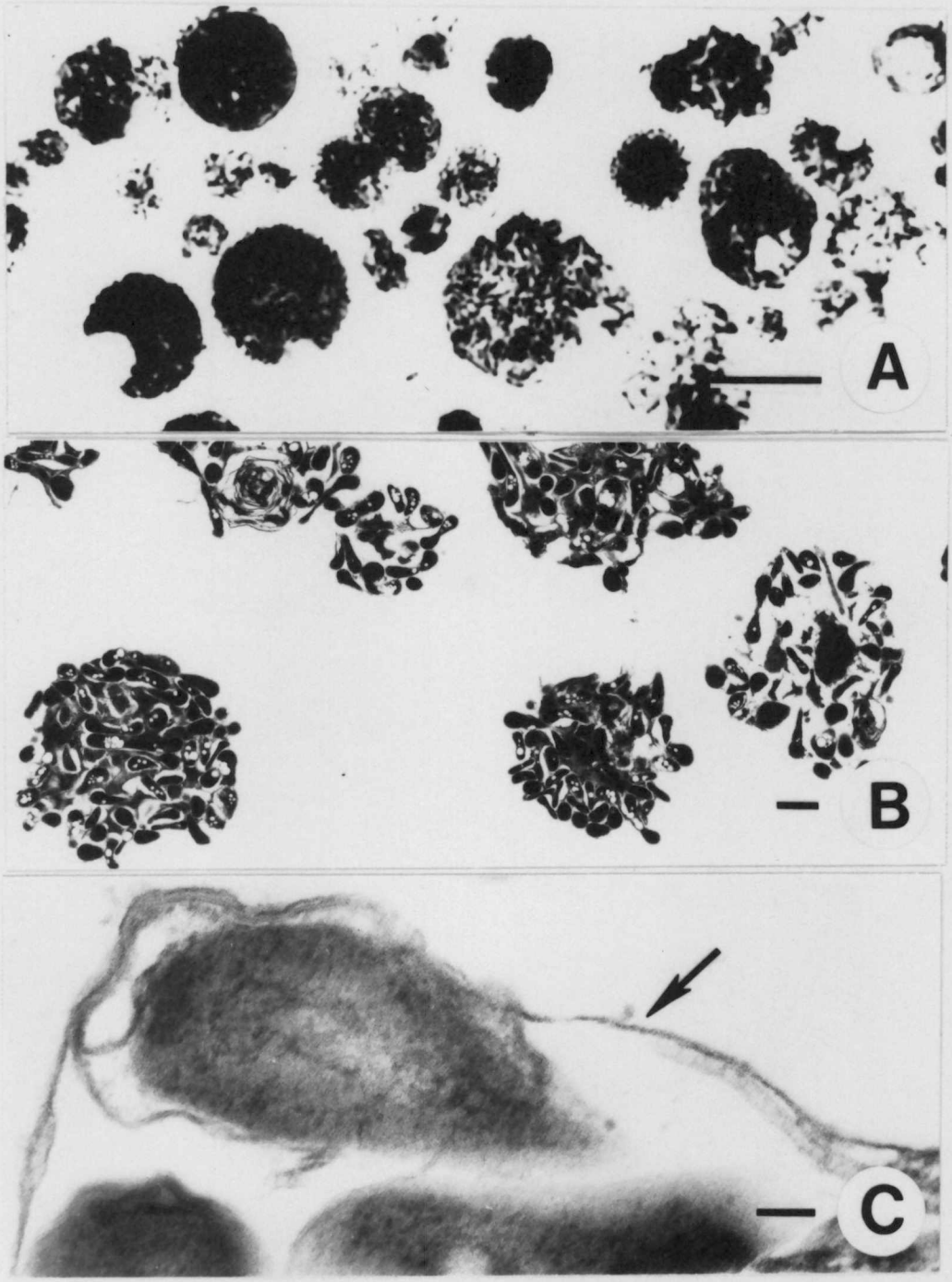

Figure 14

isolated symbiotic vacuoles. There was no difference in the purity of both preparations detectable under the phase-contrast microscope. The electrophoretic pattern of both preparations was the same.

5. Iodination of Subcellular Fractions

Cellular organelles could not be iodinated well before purification. The iodinated specific activity was low when they were iodinated in cell homogenates or in crude preparations. The cell homogenate appeared to contain a certain factor inhibiting iodination. After purification of subcellular organelles, the samples were iodinated much better. By a routine iodination procedure, the iodinated specific activity of phagosomes ($1-4x10^8$ cpm/mg protein) was higher than that of the plasmalemma ($1x10^7$ cpm/mg protein). The symbiotic vacuoles were iodinated with a lower specific activity ($1-2x10^6$ cpm/mg protein) than purified bacteria ($3-6x10^6$ cpm/mg protein). When the intact cells were iodinated, the pellet(1400 g, 20 min) of the cell homogenate had an iodinated specific activity of $8x10^5$ cpm/mg protein.

6. SDS PAGE of Subcellular Fractions

The iodinated subcellular fractions were run for SDS PAGE for 9-11 hours. Radioautograms (RAs) were prepared from the same stained gels after various time periods of exposure to obtain optimal banding patterns. The molecular weight references used were bovine serum

albumin (BSA), (m.wt. 68,000) ovalbumin (OVA), (m.wt. 43,000) and chicken muscle extract (kindly provided by Dr. Roland Bagby) which contained myosin heavy chain, α-actinin, and tropomyosin whose molecular weights were 200,000, 100,000, and 36,000 respectively.

The banding patterns of plasmalemma and phagosomes are seen in figures 15 and 16. The three PAS-stained bands of the plasmalemma in the spacer gel are labeled PAS 1. These three bands were not stained by BCB. Since they were not stained by BCB, they were likely to be mucopolysaccharides. The other three PAS-stained bands whose molecular weights were 175,000, 165,000 and 155,000 were labeled PAS II. These three bands were glycoproteins. The other BCB-stained bands of plasmalemma are labeled 1-4. With a shorter running time, there was another band between the dye front and the band '4'. The differences in banding patterns among the plasmalemma, 1 hour- and 2 hour-old phagosomes are clearly shown in figure 15. There was a significant decrease in the stained density of PAS I and PAS II bands in phagosomes. On the other hand, the two PAS-stained bands in phagosomes had molecular weights of 83,000 and 77,000 (PAS P). The stained density of these bands increased with incubation time (see PAS P of c). The BCB-staining bands 1 and 4 were lost or reduced in phagosome samples. The unique bands that are shown on the phagosome samples are labeled P1, P2, and P3.

When the radioautogram was prepared, there were some changes in the banding pattern (Fig. 16). On the corresponding position of the

Figure 15. SDS PAGE of purified plasmalemma and phagosomes

Samples

R:	molecular weight references	
A,a:	plasmalemma	60 µg protein/well
B,b:	one hour-old phagosomes	60 µg protein/well
C,c:	two hour-old phagosomes	60 µg protein/well

Briliant coomassie blue-stained (BCB) bands of the plasmalemma are labeled 1-4. PAS-staining (PAS) bands of the plasmalemma are labeled PAS I and PAS II. The unique bands of phagosomes are labeled P1-P3 and PAS P for BCB and PAS, respectively.

Note changes in the PAS-staining patterns of phagosomes. The staining density of PAS P of 2-hour old phagosomes (c) is higher than that of 1 hour-old phagosomes (b). Also note changes in the BCB-staining pattern in bands 1 and 4.

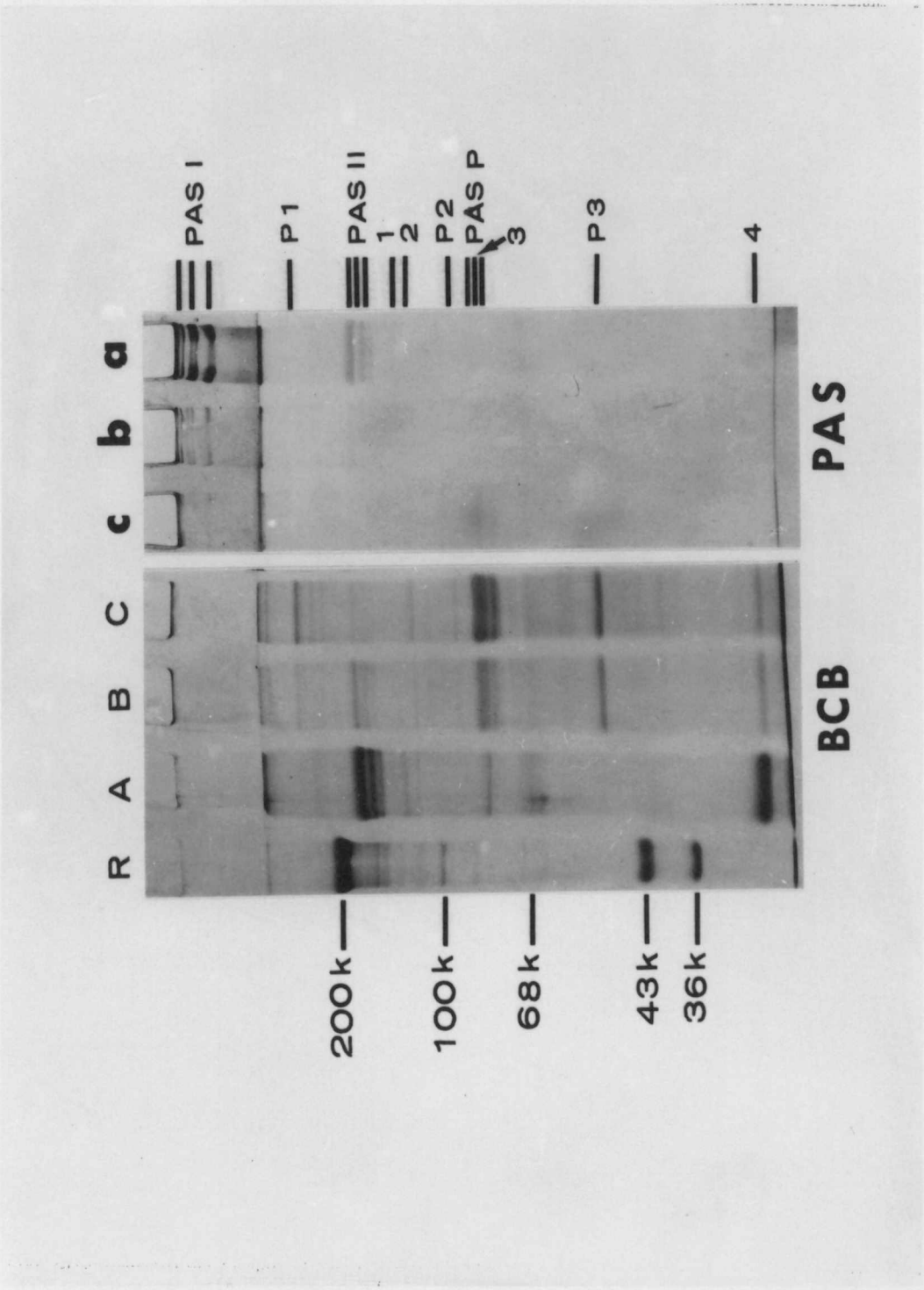
R A B C c b a
PAS I
P1
PAS II
1
2
P2
PAS P
3
P3
4
200k
100k
68k
43k
36k
BCB
PAS

Figure 15

Figure 16. Gel radioautograms of the cell membranes and phagosomes

Samples

R:	molecular weight references	
A,a:	supernatant fraction of LPO-iodinated cells, 220 µg protein /well	
B,b:	pellet fraction of LPO-iodinated cells 120 µg protein /well	
C,c:	plasmalemma	60 µg protein/well
D,d:	one hour-old phagosomes	60 µg protein/well
E,e:	two hour-old phagosomes	60 µg protein/well

Bands which are shown afer radioautography are labeled band 5 for plasmalemma, bands P4, P5, and P6 for phagosomes. Note that bands 4,2,and PAS I are not iodinated. Also note the iodinated single band on the pellet (b) of LPO-iodinated cells. None of the BCB-stained bands of supernatant fraction (A) is iodinated (a).

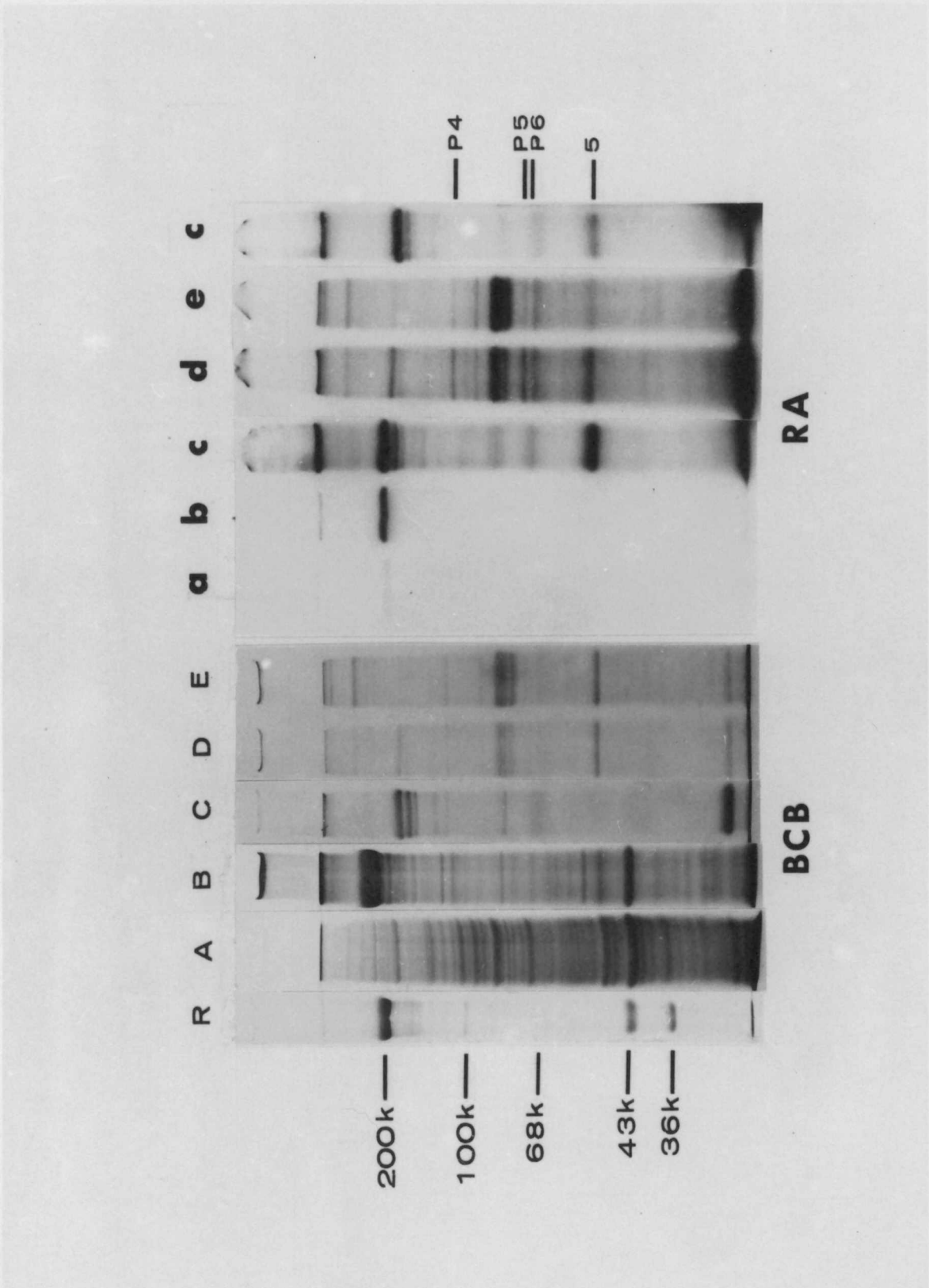

Figure 16

BCB-stained gel of the plasmalemma, the band labeled '5' was not visible (cf. C and c). This protein appeared to be small in quantity, but was readily iodinated. The molecular weight of this band was exactly the same as that of P3 band of the phagosome. When the iodinated intensity was compared, the proteins on these two bands were not the same. Another feature to note in this figure was the single iodinated band on the 'b' gel. This showed that the method of LPO-iodination was side specific and that there was only one band which could be iodinated from the outer cell surface of amoebae.

The banding patterns of SDS PAGE of phagosomes and symbiotic vacuoles are compared in figures 17 and 18. Among the BCB-stained bands of symbiotic vacuoles and bacteria, there was only one band difference (S). None of those bands of the two samples was significantly stained by PAS. The PAS-banding patterns of the plasmalemma and symbiotic vacuoles were different (Fig. 17).

In the radioautogram, there was clear difference in the major banding pattern of symbiotic vacuoles and purified bacteria. The iodinated unique bands of symbiotic vacuoles are labeled IS 1, IS 2, and IS 3. Those of bacteria are labeled IB 1 and IB 2. The faintly iodinated band on 'd' gel at IS 1 position was not detectable on the BCB pannel. Among the iodinated bands of symbiotic vacuoles, IS 2 and IS 3 bands were the same as those of the plasmalemma. It appeared possible that these two bands were due to the contaminating small cell membrane fragments in the symbiotic vacuole fraction (Chap. III,4). The

Figure 17. SDS PAGE of purified plasmalemma, symbiotic vacuoles and bacteria

Samples

R:	molecular weight references	
A,a:	plasmalemma	60 µg protein/well
B,b:	symbiotic vacuoles	120 µg protein/well
C,c:	symbiotic bacteria	120 µg protein/well
D,d:	symbiotic bacteria	120 µg protein/well

Only one unique BCB-stained band (S) could be seen.

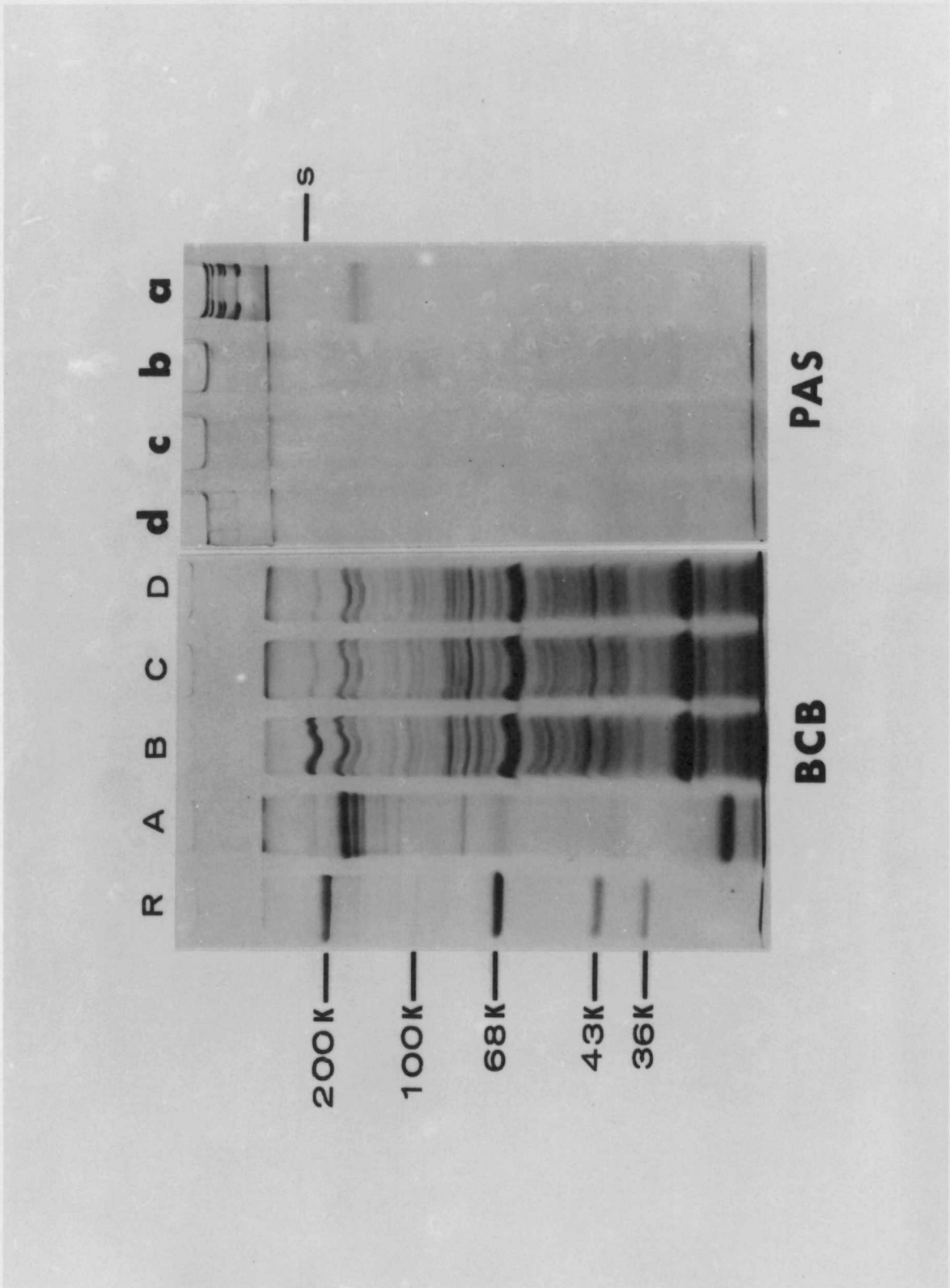

Figure 17

Figure 18. Gel radioautograms of plasmalemma, phagosomes, symbiotic vacuoles and bacteria

Samples

R:	molecular weight references	
A,a:	plasmalemma	60 µg protein/well
B,b:	one hour-old phagosomes	60 µg protein/well
C,c:	symbiotic vacuoles	120 µg protein/well
D,d:	symbiotic bacteria	120 µg protein/well

Iodinated bands of symbiotic vacuoles are labeled IS 1, IS 2, and IS 3. Iodinated bands of bacteria are labeled IB 1, and IB 2.

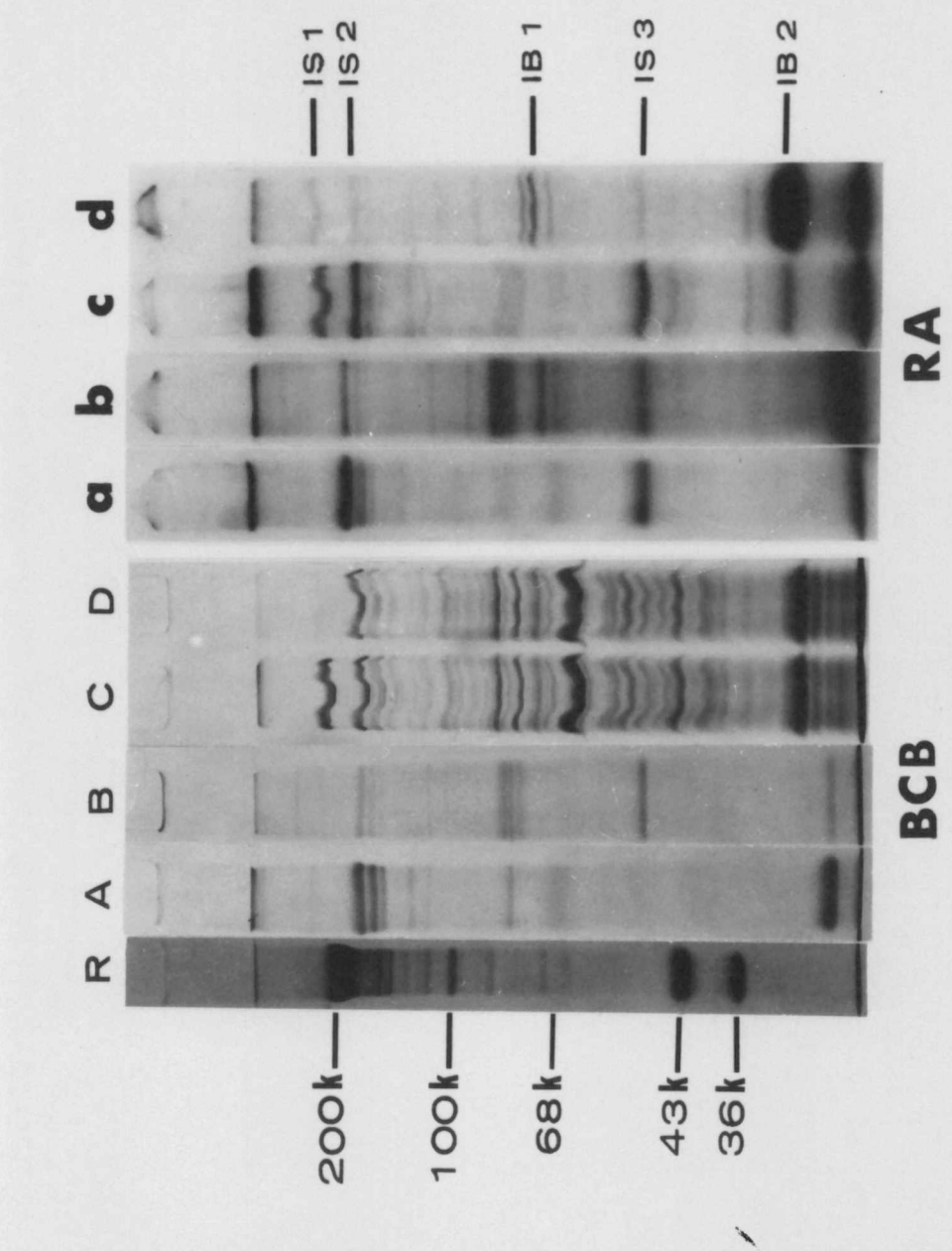

Figure 18

plasmalemma was iodinated with a specific activity ten times higher than that of symbiotic vacuoles (Chap. III,5). When 5 µg protein of the plasmalemma were added to 120 µg protein sample of bacteria, these two bands appeared in the radioautogram. There was no way to eliminate this minor contaminant in the isolation procedure. It was also difficult to quantitate the amount of contaminants by biochemical method since there was no specifically sensitive biochemical markers for the plasmalemma. If the two bands (IS 2 and IS 3) were due to contaminants, IS 1 would have been the only unique to the symbiotic vacuole membranes.

Thus, the analysis by SDS PAGE showed that there was a significant modification in molecular components of the plasmalemma following endocytosis. There was a unique protein in the symbiotic vacuole membranes (IS 1).

7. Assay of Marker Enzymes

In order to determine the optimal pH for assaying each marker enzyme, the pH profile of each enzyme was plotted after assaying its activity in the whole amoeba homogenates (Fig. 19). At pH 4.5, AcPase activity was maximum with p-nitrophenolphosphate as the substrate. Changing the pH alone strongly inhibited the enzyme activity. The AcPase activity of amoebae was not inhibited by 1 mM Na-K tartrate that had been used as an inhibitor in other cell systems. Potassium fluoride was a more effective inhibitor than Na-K tartrate (Fig. 20). The optimal pH for ATPase was 7.0-8.5. Activities of alkaline

Figure 19. A pH profile of marker enzymes in xD amoebae

The buffer systems used were as follows;
pH 4.0 - 5.5, 0.1 M Wapole's acetate buffer
pH 5.5 - 8.5, 0.1 M Tris-maleate buffer
pH 8.5 - 11.0, 0.1 M glycine-NaOH buffer

A. Acid and alkaline phosphatases
p-Nitrophenol phosphate was used as the substrate.
B. Alkaline phosphodiesterase
p-Nitrophenyl N-acetyl -D glucosaminide was the substrate

C. 5'-Nucleotidase. 5'-AMP was the substrate.
D. $(Na^{+}-K^{+})$-ATPase. ATP was the substrate.
E. Mg^{2+}-ATPase. ATP was the substrate.
F. Glucose 6-phosphatase. G6P was the substrate.

Absorbance was taken at 410 nm for A and B, and at 660 nm for C, D, E, and F. The figure is one of the typical pH profiles among those obtained from three enzyme assays.

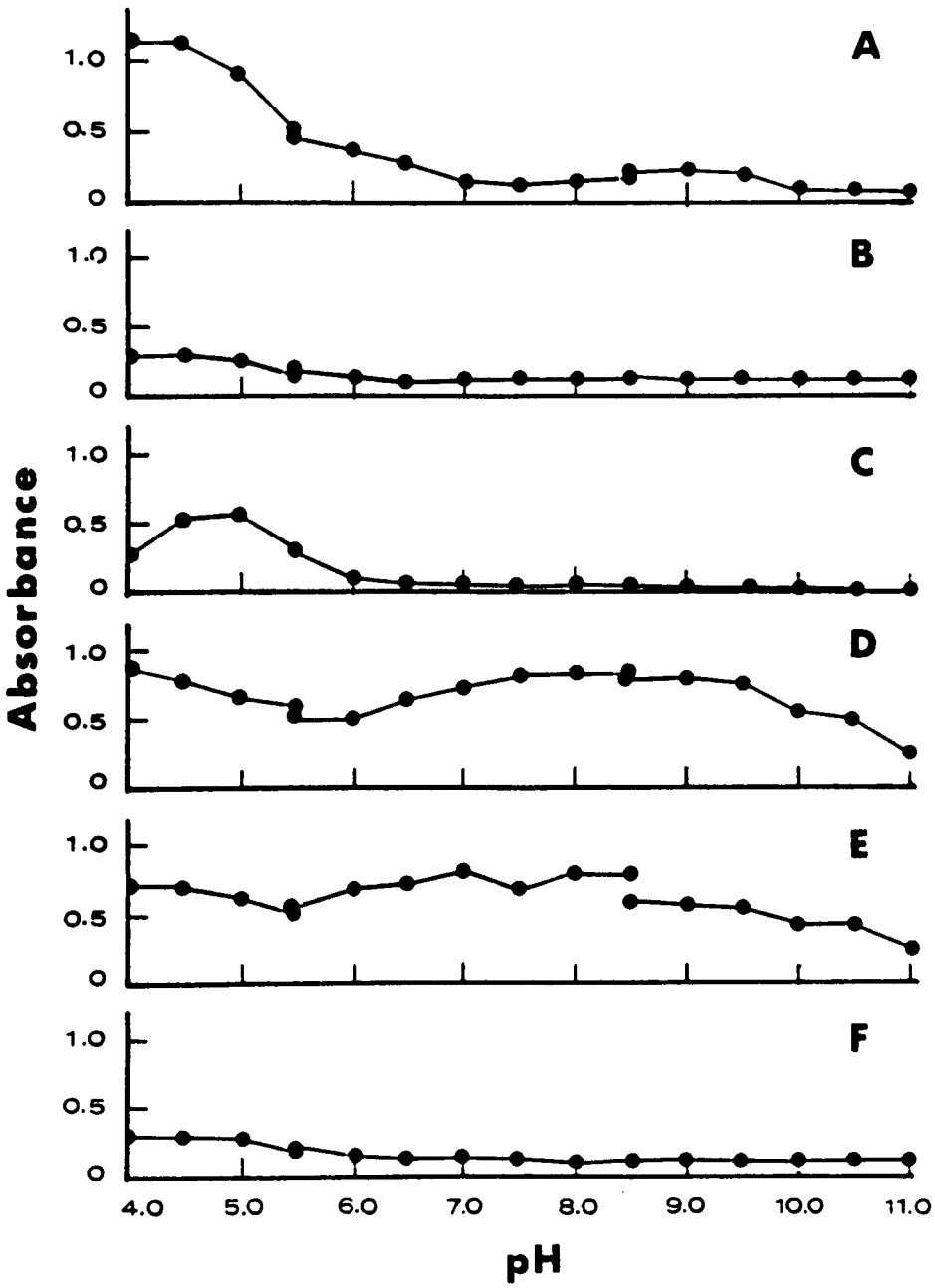

Figure 19

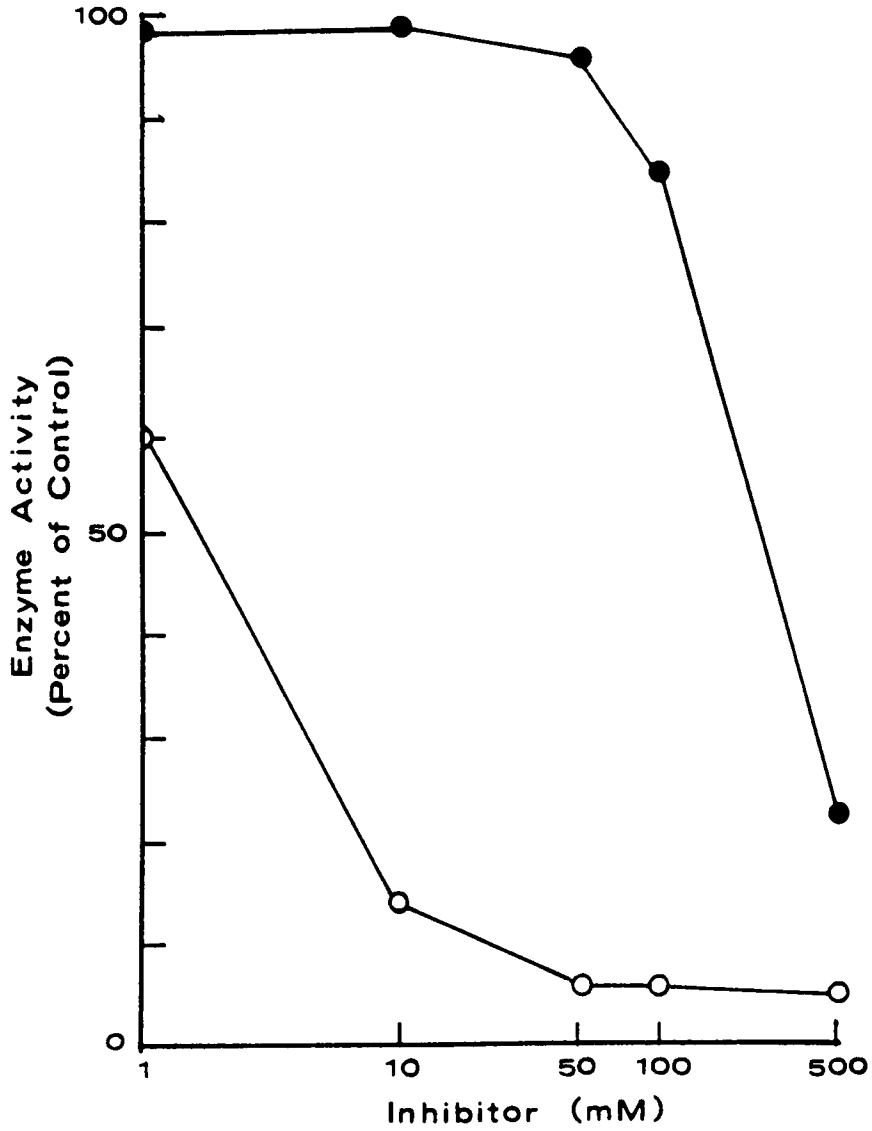

Figure 20. Inhibition of AcPase of xD amoebae

Solid circles, Na-K tartrate as an inhibitor
Open circles, KF as an inhibitor.

phosphodiesterase and 5'-NTPase were not detectable in the whole homogenate of amoebae. G6Pase activity was also low and did not form a peak in the pH profile.

The results of enzyme assay of subcellular fractions are summarized in table 1. The AcPase activity in phagosomes was about twice that in the cell homogenate. None of the commonly known marker enzymes of plasma membranes, (Na^+-K^+)-ATPase, 5'-NTPase, or alkaline phosphodiesterase, was present in the the plasmalemma fraction of amoebae. The apparent (Na^+-K^+)-ATPase activity was higher in the cell homogenate than in the plasmalemma fraction. To determine if this was due to the true (Na^+-K^+)-ATPase, the effect of ouabain, a specific enzyme inhibitor, was studied (Fig. 21). The ATPase was not inhibited by ouabain, even at the 15 mM concentration. The enzyme activity was not altered by varying the Na^+/K^+ ratio of the incubation medium (Fig. 22A). There was a significant enhancement in the enzyme activity by increasing Mg^{2+} concentration up to 3 mM (Fig. 22B). The apparent (Na^+-K^+)-ATPase activities in cell homogenate and symbiotic vacuoles were not due to the true (Na^+-K^+)-ATPase.

The specific activity of G6Pase was low overall, but the enzyme activity was higher in the cell homogenate than in any other subcellular fractions.

TABLE 1

ENZYME ASSAY OF SUBCELLULAR FRACTIONS OF xD AMOEBAE

Enzymes	AcPase	Mg^{2+} ATPase	$(Na^{+}-K^{+})$ ATPase	AMPase	G6Pase	AlkPDEase NPTP	AlkPDEase NPAGA
Cell Homogenates	76.3±2.05*	79.4±5.25	73.5±16.91	3.1±0.33	26.4±6.77	0.0	0.1±0.10
Plasmalemma	17.3±3.02	15.1±2.14	4.1±1.41	3.8±1.56	2.9±1.15	0.1±0.00	0.4±0.07
Phagosomes	165.3±13.93	45.2±15.78	17.1±3.08	6.1±2.41	1.5	0.1	1.0
Symbiotic Vacuoles	65.6±9.64	43.7±4.15	116.0±23.20	1.4±0.78	10.1±1.61	0.7±0.25	1.9±0.21
Symbiotic Bacteria	31.2±4.15	46.0±10.84	12.1±7.89	0.3±0.29	2.6±0.94	0.3	1.9

*The enzyme activity (nmol Pi/mg protein/min) is shown as mean ± s.e.m. of 4-14 assays. Alkaline phosphodiesterase (AlkPDEase) was assayed with either p-nitrophenyl thymidine-5'-phosphate (NPTP) or p-nitrophenyl N-acetyl D-glucosaminide (NPAGA) as the substrate.

Figure 21. Inhibition of ATPase of xD amoebae by ouabain

Figure 22. The effect of ionic composition on ATPase activity

A. The effect of K^+/Na^+. In the assay system, the ratio of K^+/Na^+ (mM/mM) was changed, but the ionic strength remained constant in all assay.

B. The effect of Mg^{2+}.

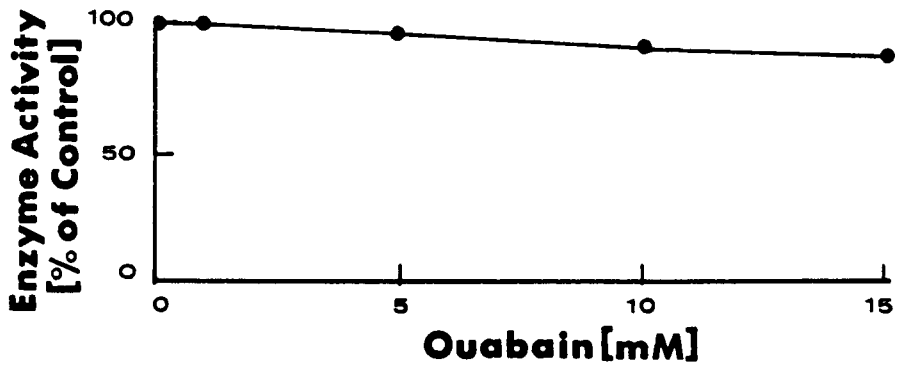

Figure 21

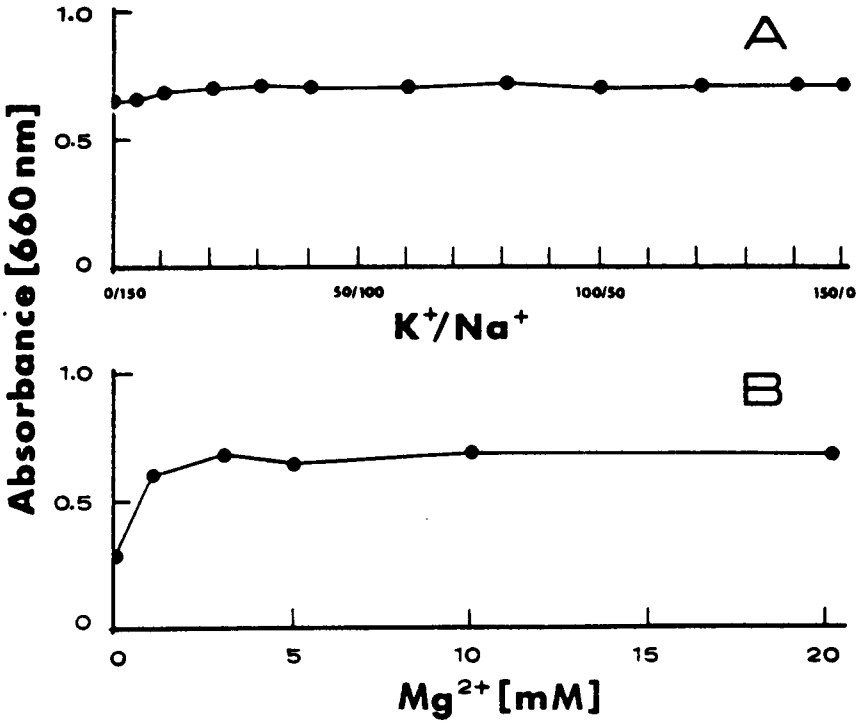

Figure 22

CHAPTER IV

DISCUSSION

Subcellular Fractionation of xD Amoebae

The objective of this study was to detect any structural or functional differences between the phagosomes and symbiotic vacuoles. Fluorescence microscopy of AO-labeled cells and electron microscopy of ferritin-labeled xD amoebae showed that the absence of AcPase activity in symbiotic vacuoles was due to the inhibition of lysosomal fusion (Chap. III,1).

In studying the biochemical characteristics of these vacuoles, the initial step was the purification of subcellular organelles. This was done by using a newly developed procedure in which nylon screens were utilized. The amoeba is a highly compartmentalized organism with many subcellular organelles (Allen et al., 1974), and the characteristics of crystals and the cell surface make it difficult to purify the plasmalemma and symbiotic vacuoles. First, the apparent high density of the nuclei, plasmalemma, and crystals cause them to pellet together at the bottom of the tube after centrifugation for 10 min at 4000 g even in 60 % (w/w) sucrose. This problem had also been noted in an earlier study of the cell surface of amoebae (Allen et al., 1974). The authors considered the contamination by small quantities of granules and crystals unavoidable. The apparent density of the plasmalemma of the amoeba is not comparable to those of other protozoans or mammalian cells

(cf. HeLa cells, Boone et al., 1969; rat liver, Henning et al., 1970; Acanthamoeba, Ulsmer et al., 1971). So the conventional methods which had been developed for other cell systems (Brunette & Till, 1971; Warren & Glick, 1971) were not directly applicable to amoebae. Second, the size of the fragmemted membranes was big after gentle sonication at the intensity with which the cells were broken, but the nuclei and symbiotic vacuoles were intact (Chap. II,3). The trapped contaminants by large membrane fragments were difficult to remove by centrifugation. Third, symbiotic vacuoles were fragile and ranged from a few to 20 µm or more in size. To improve the yield of symbiotic vacuoles, the isolation had to be completed soon after homogenization. The symbiotic vacuoles were intact till 50 min after homogenization in 10 % buffered sucrose solution. The best approach in solving the problems was to remove the crystals that ranged 1-2 x 3-5 µm in size (Stockem, 1978) or to separate the membrane fragments from the crystals. During a preliminary experiment of the subcellular fractionation, a 5 µm-pore screen was used to filter out the crystals. Due to the small pore size, the flow rate of the screen was extremely low even with pressure applied on the filter and the pores were pluged up by large cell organelles. Instead, by using a 30 µm-pore screen, all of the small cellular organelles including crystals were filtered leaving large membrane fragments and nuclei on top of the screen. Another advantage of using a 30 µm-pore screen was the convenience to wash the membrane fragments simply by spraying the buffer solution from the top of the screen. The symbiotic

vacuoles in the filtrate were also readily purified by one step centrifugation on a sucrose step gradient. So, the fragile symbiotic vacuoles could be fractionated in 30 min after sonication.

The new procedure has several advantages as compared with previously used procedures for isolating the plasmalemma of amoebae (O'Neil, 1961, 1964; Allen et al., 1974). With this procedure, the contaminating granules and crystals can be removed completely. The procedure can be completed in a few hours or overnight. In the electron microscope the glycocalyx of the purified plasmalemma was still visible though the shape was irregular when compared with the structure on an intact cell. Due to the absence of known chemical or enzymatic markers for the amoeba cell surface (Allen et al., 1974; Chap. III,3), the purity of this preparation could not be compared quantitatively with others. In ultrastructure, the presence of the glycocalyx on the outer surface was the unequivocal marker of the plasmalemma (O'Neill, 1964).

After gaining the purified membrane fractions, it was hoped to detect a certain functional marker. As is shown in the results of the enzyme assay in the table (Chap. III,7), none of the known plasma membrane markers was detectable in the cell membranes. The negative results indicated the functional simplicity of the amoeba cell surface (Allen et al., 1974). The amoeba cell surfaces are not known to have any catalyzed transmembrane transport process (Chapman-Andresen & Holter, 1955). Both the compositional and the functional simplicity of the amoeba cell surface relative to the plasma membranes of mammalian

cells may be the consequence of a highly specialized structure for endocytosis (Korn, 1975).

Cytochemistry of marker enzymes showed that none of the marker-enzyme activities was associated with the membranes of symbiotic vacuoles , phagosomes, or plasmalemma. Stockem and Klein (1979) showed the presence of calcium-binding sites on the plasma membrane of amoebae. In the ultrastructural study it was not clear if the binding sites (93 nm in diameter of the electron-dense granules) were molecules in the membrane or existed in simple association with the cytoplasmic surface of the plasmalemma. An experiment was done in the present study to see if these binding sites were also present in the periphery of the phagosomes. When the cells were fixed in the presence of 5 mM $CaCl_2$ in the aldehyde fixative (Stockem & Klein, 1979), most of the cytoplasm was badly washed away, and the phagosomes and symbiotic vacuoles were not intact morphologically.

The specific activity of AcPase in the two-hour-old phagosome fractions was more than twice that of the cell hommogenates. The presence of AcPase activity in the phagosomes showed a good correlation with the morphological change of the phagosomal membranes and modification of the banding patterns of SDS PAGE (see Figs. 15 and 16). The symbiotic vacuoles had much higher (Na^+-K^+)-ATPase activity than phagosomes or the plasmalemma. This enzyme activity appeared to be due to the presence of symbiotic bacteria. Pure subcellular fractions were made by the newly developed procedure, but there were no detectable functional markers in any of those vacuole membranes or the plasmalemma.

Morphological and Compositional Modification of Phagosomal Membranes

A significant correlation has been found between the ultrastructural alterations and the protein-pattern changes of the phagosomal membranes during cellular digestion. Amoebae attached to the surface of latex beads by the typical glycocalyx of the outer surface of the plasmalemma. The cells made phagosomes by a simple invagination of the membranes surrounding the beads. A distinct alteration in the ultrastructure of the phagosomal membrane is shown in the figure 7C. A loss of the mucus layer of the phagosomes had also been noted by a simple morphological examination (Nachmias & Marshall, 1961), or by the lack of PAS reaction after cytochemical staining (Jacques, 1969).

In this study, the results of SDS PAGE of the plasmalemma and phagosomes showed that there was a significant modification of the component molecules of the membranes accompanying morphological changes (see Figs. 15 and 16). There were three PAS-staining bands in the spacer gel of plasmalemma (PAS 1, Fig. 15). None of these three bands was stained by BCB. The molecules in these three PAS-staining bands appeared to be the components of the glycocalyx which is composed of a mixture of neutral and acid mucopolysaccharides (Chapman-Andresen, 1965). The other three bands of the PAS II of the plasmalemma were glycoproteins. This is the first demonstration of glycoprotein in the gel electrophoresis of plasmalemma. The two PAS-staining bands of phagosomes (PAS P) were also glycoproteins. The PAS P bands were likely

to be modified glycoproteins of the plasmalemma (PAS II). All three glycoprotein bands of PAS II seemed to undergo a modification following lysosomal fusion. In addition to the glycoproteins, there were some other changes in BCB-banding patterns of the plasmalema following phagolysosome formation (Figs. 15 and 16). So, the modification of the plasmalemma following phagocytosis appeared to be due to more than a simple loss of glycocalyx. At present there are no detectable functional markers on the phagosomal membranes. Such a modification of the compositional molecules of the phagosomal membrane has been considered to bring about changes in the permeability of membranes, or to turn on non-functional carriers or enzymes (Chapman-Andresen & Holter, 1955).

The results of this study on amoebae differ significantly from those on cultured macrophages obtained in Cohn's laboratory (Muller et al., 1979, 1980a, 1980b). In their studies, the molecular compositions of the cell membranes and phagosomes of the macrophage were compared by SDS PAGE. The luminal side of the phagosomes was iodinated by LPO which was conjugated to latex beads, and the plasma membranes were iodinated from the outer surface. Minor differences were found in the banding pattern of radioautograms between the homogenates of iodinated cells and the phagosomes that had been iodinated one hour after phagocytosis. From these studies, the authors drew the following conclusions: There are no novel proteins which can be detected in phagosomes either immediately after phagosme-lysosome fusion or after one hour of

intra-cytoplasmic residence (Muller et al., 1980). The proteins of phagosome membranes can return intact, i.e., recycle, to the cell surface (Muller et al., 1979). There is a continuous bidirectional flow of membrane polypeptides between the plasma membrane and phagolysosomes.

Since the membrane charcteristics of mammalian cells are very different from those of protozoans (Korn, 1975), such different results and conclusions are possible. But there are a few comments to be made on their experiments. First, the efficiency of iodination from the luminal side of the phagosomes may be limited after lysosome fusion. Second, they made comparisons only on radioautograms as they did not purify the phagosomes or the cell membranes. In SDS PAGE of the present study (Chap. III,6), one hour-old phagosomes still contained the major membrane components (see PAS I, PAS II bands), whereas the banding pattern of the two hour-old phagosomes was significantly different from that of the plasmalemma.

Structural and Functional Aspects of the Symbiotic Vacuole Membranes

In this study some of the compositional and functional differences between symbiotic vacuoles and phagosomes were shown by SDS PAGE and assay of subcellular fractions. As the bacteria-containing vacuoles were surrounded by a single unit membrane, the presence of vacuole membrane could not be detected under the light microscope after rupturing symbiotic vacuoles. The membranes of symbiotic vacuoles also

did not have any chemical or biochemical markers to trace. Isolation of the vacuole membranes was attempted after ^{125}I-iodination to trace the labeled membranes by sucrose linear- or step-gradient centrifugation. In this experiment, there were two problems that made it difficult to trace the labeled markers: First, the iodinated specific activity of the symbiotic vacuole was very low (Chap. III,5). Second, even after washing by centrifugation and extensive dialysis, the radioactivity of unincoporated free iodine of the symbiotic bacteria obscured that of the barely labeled vacuole membranes in the gamma counter. The banding patterns of the two kinds of vacuoles and the plasmalemma could be compared only by radioautography (Fig. 18). Symbiotic vacuoles had a unique protein that could be labeled from the cytoplasmic surface of the vacuole (IS 1). There was a distinct difference in overall banding pattern of these two vacuole fractions. Such a difference in the pattern of iodinated proteins of vacuoles is quite possible since the membranes of symbiotic vacuoles are adapted to support the endocellular symbiosis of bacteria. The unique protein of symbiotic vacuoles may be one of the important functional or structural molecules in maintaining symbiosis. As the fusion mechanism or the control of lysosomal fusion is not known, it is difficult to explain the role of the IS I protein. As reviewed in Chapter I, there has been no study done to compare structural or functional characteristics of the parasitic or symbiotic vacuole membranes. The study of phagolysosome formation is also far from being complete to give any definitive answers for the fusion

mechanism in terms of physiology or biochemistry of vacuole membranes.

In the classic approach in pathology, released metabolites from an infective organism were considered as inhibitors of lysosomal fusion (Jones & Hirsch, 1972; Hirsch et al., 1974). In the study of lysosomotropic agents, however, the fusion has been thought to be controled by the lysosomes rather than by factors from endocytosed material (Hart & Young, 1975, 1979; Geisow et al., 1980). In the study of conjugated chloride-oxidized amylose (COAM), Geisow et al., (1980) proposed that the control site of lysosomal fusion was on lysosomes rather than on phagosomes. The conjugated COAM with fluorescent label or with an electron-opaque label was found in the secondary lysosomes. The COAM-containing secondary lysosomes did not fuse with phagosomes. It has been hypothesized that the inhibitory mechanism might be perturbation of the transport process of the lysosomal membranes (Goren et al., 1976; Hart & Young, 1979), or more likely alteration of the distribution or physical state of membrane components (Geisow et al., 1980). Another possible control of lysosomal fusion has been proposed from the study of phagolysosomal fusion in vitro (Oates & Touster, 1980). From a series of study, Oates and Touster (1980) concluded as follows; "The rate of phagolysosomal fusion in vitro of Acanthamoeba might be regulated by cyclic nucleotides, with enhancement of the fusion rate by cAMP and inhibition of the rate by cGMP. The extent of fusion increase by conversion of significant fraction of the vacuole population from a fusion-incompetent to a fusion-competent, cyclic

nucleotide-sensitive state." The functional mechanism of the cyclic nucleotides or the conversion mechanism is not known, however.

The latter two groups of studies of the above indicate that the component molecules of vacuole membranes may be involved in the control of phagolysosomal fusion. Rikishia and Mizuno (1978) also emphasized the importance of the membrane component for modifying the efficiency of lysosomal fusion.

Further characterization of the unique proteins of the symbiotic vacuole membranes in xD amoebae will give further insight to the understanding of the mechanism of lysosomal fusion and the maintenance of endocellular symbiosis.

The Amoeba as a Model for Membrane Studies

The present study has confirmed the simplicity of the plasmalemma both in its molecular composition and function. Among the visible bands of three mucopolysaccharides, three glycoproteins, and six proteins detected by SDS PAGE, only one glycoprotein band (m.wt. 175,000) was iodinated by LPO from the outside of the cell surface (Fig. 16). Such a simplicity of the membrane composition would be an advantage in studying the membrane flow and the fate of membrane components accompanying cellular activities. This unique glycoprotein (m.wt. 175,000) could be used as a marker to study the fate of membrane components. The glycoprotein band was one of the bands which appeared to undergo alteration during phagocytosis and cellular digestion. By

using this glycoprotein as a marker, an indirect evidence could be obtained for the presence of receptor sites or for the involvement of specific membrane components in cellular endocytosis. After labeling the glycoproteins from the outside of the cell surface by LPO, the presence of labels in phagosomal membranes or plasmalemma could be compared quantitavely by radioautography on thin sections and by measuring the radioactivity after subcellular fractionation.

In addition to the extensive alteration of the banding pattern in SDS PAGE of phagosomes, component molecules of the glycocalyx of phagosomal membranes were lost by cellular digestion (Figs. 7C, 15; Nachmias & Marshall, 1961). The functional aspect of the glycocalyx is not known, but it may play a role in the adhesion of cell surface to other objects. The formation of the fringes in amoebae has been shown by electron microscopy (Wise & Flickinger, 1970b), but the mechanism for the transfer of molecular components from phagosomes to their synthetic sites of fringes is not known. This may be another interesting subject to study by utilizing this cell system. The sugar moiety of the glycocalyx can be specifically labeled from the outside of the cell surface by galactose oxidase and tritiated sodium borohydride (Gahmberg & Hakomori, 1973). The fate of the ^{3}H-labeled sugar moiety can then be followed by radioautography.

Significant differences exist between the membranes of phagosomes and those of symbiotic vacuoles, as can be demonstrated by gel electrophoresis of LPO-iodinated samples. Since the vacuole membranes

surrounding bacteria in the experimentaly infected amoebae are originally derived from the phagosomal membranes (Ahn & Jeon, 1979), it would be interesting to follow the modification of component molecules in the phagosomal membranes during their transition to symbiotic vacuoles. It would be possible to follow the alteration of membrane components of the vacuoles by using traceable membrane markers for the two kinds of vacuoles (phagosomes and symbiotic vacuoles). In addition, since the properties of symbiotic bacteria in xD amoebae are very similar to those of pathogenic bacteria (cf. Chapter I), the xD-amoeba system could serve as a model for the study of endocellular parasitism and symbiosis. Results obtained from such studies would be useful in understanding organismic association in general, including the infection of mammalian cells by microorganisms such as Toxoplasma and Mycobacterium. The results presented in this dissertation demonstrate that the amoeba can serve as a useful model for studies of membrane dynamics.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ahn, T. I. & K. W. Jeon (1979) Growth and electron microscopic studies on an experimentally established bacterial endosymbiosis in amoeba. J. Cell. Physiol. 98, 49-58.

Allen, J. M. (1969) Lysosomes in bacterial infection. In "Lysosomes in Biology and Pathology" v.2, 41-68.Ed by J.T.Dingle and H.B.Fell, North Holland Publ. Co. London and Amsterdam.

Allen, H.J., C.Ault., R.J.Winziler, & J.F.Danielli (1974) Chemical characterization of the isolated cell surface of amoeba. J. Cell Biol. 60, 26-38.

Armstrong, J. A & P. D. Hart (1971) Response of cultured macrophages to Mycobacterium tuberculosis, with observation on fusion of lysosomes with phagosomes. J. Exp. Med. 134, 713-740.

Beaufay, H., A.Amar-Costesec, E.Feytmans, D.Thines-Sempoux, M.Wibo & M.Robbi (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver I. Biochemical methods. J. Cell Biol. 61, 188-200.

Boone, C.W., L.E.Ford, H.E.Bond, D.C.Stuart, & D.Lorenz (1969) Isolation of plasma membrane fragments from HeLa cells. J. Cell Biol. 41, 378-392.

Bower, B (1980) A morphological study of plasma and phagosome membranes during endocytosis in Acanthamoeba. J. Cell Biol. 84, 246-260.

Brunette, D. M. & J. E. Till (1971) A rapid method for the isolation of L-cell surface membranes using an aqueous two phase polymer system. J. Memb. Biol. 5, 215-224.

Casley-Smith, J. R. & T. Savanat (1966) The formation of membranes around microorganisms and particles injected into amoebae; Support for the 'reticulosome' concept. Aust. J. Exp. Biol. Med. Sci. 44, 111-122.

Chang, K. P. & D. M. Dwyer (1976) Multiplication of a human parasite (Leishmania donovani) in phagosomes of hamster macrophages in vitro. Science 193, 678-680.

Chang, K. P. & D. M. Dwyer (1978) Leishmania donovani. Hamster macrophage interactions in vitro: Cell entry, intracellular survival, and multiplication of amastigotes. J. Exp. Med. 147, 515-530.

Chapman-Andresen, C. (1965) The induction of pinocytosis in amoebae. Arch. Biol. 76, 189-207.

Chapman-Andresen, C. (1973) Endocytotic process. In "Biology of Amoeba" Ed. by K.W.Jeon pp. 319-348. Academic Press, New York

Chapman-Andresen, C. & S. Christensen (1970) Pinocytic uptake of ferritin by amoeba Chaos chaos measured by atomic absorption. Compt Rend. Trab. Lab. Carlsberg 38, 19-58.

Chapman-andresen, C., H. Holter (1955) Studies on the ingestion of ^{14}C glucose by pinocytosis in the amoeba Chaos chaos. Exp. Cell Res. Suppl. 3, 52.

DeDuve, C., T.DeBarsy, B.Poole, A.Trouet, P.Tulkens & F.V.Hoof (1974) Lysosomotropic agents. Biochem. Pharmacol. 23, 2495-2531.

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

Dixon, T. F., & M. Purdon (1954) Serum 5' nucleotidase. J. Clin. Pathol. 7, 341.

Fairbanks, G., T.L.Steck, & F.H.Wallach (1971) Electrophoretic analysis of the major polypeptides of the erythrocyte membrane. Biochem. 10, 2606-2630.

Farquhar, M. G., J.J.M.Bergerron, & G.E.Palade (1974) Cytochemistry of Golgi fractions prepared from rat liver. J. Cell Biol. 60, 8-25.

Fiske, C. H. & Y Subbarow (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-401.

Fris, R. R. (1972) Interaction of L cells and Chlamydia psittaci: Entry of the parasite and host responses to its development. J. Bacteriol. 110, 706-721.

Gahmberg, C. G. & S. Hakomori (1973) External labeling of cell surface galactose and galactoseamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem. 248, 4311-4317.

Geisow, M. J., P.D.Hart, & M.R.Young (1980) Site of action of phagosome-lysosome fusion in cultured macrophages. Exp. Cell Res. 126, 159-165.

Goldstein, L. & C. Ko (1976) A method for the mass culturing of large free-living amoebas. Meth. Cell Biol. 13, 239-246.

Goren, M. B., P.D.Hart & M.R.Young (1976) Prevension of phagosome-lysosome fusion in cultured macrophages by sulfatides of mycobacterium tuberculosis. Proc.Nat.Acad.Sci.USA 73, 2510-2514.

Hart, P. D. & M. R. Young (1975) Interference with normal phagosome-lysosome fusion in macrophages, using ingested cells and suramin. Nature 256, 47-49.

Hart, P. D. & M. R. Young (1970) The effect of inhibitors and enhancers of phagosome-lysosome fusion in cultured macrophages on the phagosome membranes of ingested yeasts. Exp. Cell Res. 118, 365-376.

Hayat, M. A. (1973) Specimen preparation. In "Electron Microscopy of Enzymes: Principles and Methods" Ed by M.A.Hayat v.1, 1-43. Van Nostrand Reinhold Co., New York

Hirsch, J. G., T.C.Jones, & L.Len (1974) Interactions in vitro between Toxoplasma gondii and mouse cells. In "Parasites in the Immunized Host" Ciba Found. Symp. 25, 205-223.

Hubbard, A. L. & Z. A. Cohn (1975) Externally disposed plasma membrane proteins I. Enzymatic iodination of mouse L cells. J. Cell Biol. 64, 438-460.

Jacques, P. J. (1969) Endocytosis. In "Lysosomes in Biology and Pathology" v.2, 395-420. Ed by J.T.Dingle and H.B.Fell North Holland Publ. Co., London and Amsterdam

Jeon, K. W. (1965) Simple method for staining and preserving epoxy resin-embedded animal tissue sections for light microscopy. Life Sci, 4, 1839-1841.

Jeon, K. W. (1972) Development of cellular dependence on infective organisms: Micrurgical studies in amoebas. Science 176, 1122-1123.

Jeon, K. W. & T. I. Ahn (1978) Temperature sensitivity: A cell character determined by obligate endosymbionts in amoebas. Science 202, 635-637.

Jeon, K. W. & J. C. Hah (1977) Effect of chloramphenicol on bacterial endosymbionts in a strain of Amoeba proteus. J. Protozool. 24, 289-293.

Jeon, K. W. & M. S. Jeon (1975) Cytoplasmic filaments and cellular wound healing in Amoeba proteus. J. Cell Biol. 67, 243-249.

Jeon, K. W. & M. S. Jeon (1976) Endosymbiosis in amoebae: Recently established endosymbionts have become required cytoplasmic components. J. Cell. Physiol. 89, 337-344.

Jeon, K. W. & I. J. Lorch (1967) Unusal intra-cellular bacterial infection in large free-living amoebae. Exp. Cell Res. 48, 236-240.

Jeon, K. W. & I. J. Lorch (1968) New simple method of micrurgy on living cells. Nature 217, 463.

Johnston, R. B. & J. E. Lehleyer (1977) The involvement of oxygen metabolites from phagocytic cells in bactericidal activity and inflamation. In "Superoxide and Superoxide Dismutase" Ed by A.M.Michelson, J.M.McCord, and I.Fridovich. Academic Press, New York

Jones, T. C. & J. G. Hirsch (1972b) The interaction between Toxoplasma gondii and mammalian cells II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. Exp. Med. 136, 1173-1194.

Jones, T. C., S.Yeh, & J.G.Hirsch (1972a) The interaction between Toxoplasma gondii and mammalian cells. I. Mechanism of entry and intracellular fate of the parasite J. Exp. Med. 136,1157-1172.

Karakashian, M. W. (1975) Symbiosis in Paramecium bursaria. Sympo. Soc. Exp. Biol. 29, 145-174.

Karnovsky, M. J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27, 137A.

Klebanoff, S. J. (1975) Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Sem. Hematol. 12, 117-142.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lowry, O. L., N.J.Rosebrough, A.L.Farr, & R.J.Randell (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Luft, J. H. (1961) Improvement in epoxy resin embedding methods. J. Biophys.. Biochem. Cytol. 9, 409-414.

Moulder, J. W. (1974) Intracellular parasitism: Life in an extreme environment. J. Infec. Dis. 130, 300-306.

Moulder, J. W. (1979) Speculation on the evolutionary origin of intracellular parasitism.. In "Host-Parasite Interfaces" Ed. by Brent B. Nickol pp 127-138.Academic Press, New York

Muller, W. A., R.M.Steinman, & Z.A.Cohn (1979) Recycling of phagolysosome membrane proteins in cultured macrophages. J. Cell Biol. 83, 258a.

Muller, W. A., R.M.Steinman, & Z.A.Cohn (1980a) The membrane proteins of the vacuolar system. I. Analysis by novel method of intralysosomal iodination. J. Cell Biol. 86, 292-303.

Muller, W. A., R.M.Steinman, & Z.A.Cohn (1980b) The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and plasma membrane. J. Cell Biol. 86, 304-314.

Muscatine, L., C.B.Cook, R.L.Pardy, & R.R.Pool (1975) Uptake, recognition and maintenance of symbiotic chlorella by Hydra viridis. Symp. Soc. Exp. Biol. 29, 175-204.

Nachmias, V. & J. M. Marshall (1961) Protein uptake by pinocytosis in amoeba: Studies on ferritin and methylated ferritin In "Biological Structure and Function" Ed by T. Goodwin & O. Lindberg v.2, 605. Academic Press, New York

Oates, P. J. & O Touster (1976) In vitro fusion of Acanthamoeba phagolysosomes I. Demonstration and quantitation of vacuole fusion in Acanthamoeba homogenates. J. Cell Biol. 68, 319-338.

Oates, P. J. & O. Touster (1978) In vitro fusion of Acanthamoeba phagolysosomes II. Quantitative characterization of in vitro vacuole fusion by improved electron microscope and new light microscope technique. J. Cell Biol.79, 217-234.

Oates, P. J. & O. Touster (1980) In vitro fusion of Acanthamoeba III. Evidence that cyclic nucleotides and vacuole subpopulations respectively control the rate and the extent of vacuole fusion in Acanthamoeba homogenates. J. Cell Biol. 85, 804-810.

O'Neill, C. H. (1964) Isolation and properties of the cell surface membrane of Amoeba proteus. Exp. Cell Res. 35, 447-496.

O'Neill, C. H. & L. Wolpert (1961) Isolation of the cell membrane of Amoeba proteus. Exp. Cell Res. 24, 592-595.

Peterson, P. K., J.Verhoef, D.Schmeling, & P.G.Quie (1977) Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. J. Infec. Dis. 136, 502-509.

Phillips, D. R. & M. Morrison (1971) Exposed protein on the intact human erythrocyte. Biochem. 10, 1766-1771.

Poste, G. & A. C. Allison (1973) Membrane fusion Biochim. Biophys. Acta 300, 421-465.

Quie, P. (1975) Pathology of bactericidal power of neutrophils. Sem. Hematol. 12, 143-160.

Raz, A. & R. Goldman (1974) Spontaneous fusion of rat liver lysosomes in vitro. Nature 247, 206-208.

Reynolds, E. S. (1963) The use of lead citrate at high pH as an electron-opaque stain for electron microscopy. J. Cell Biol. 17, 208.

Rikihisa, Y. & D. Mizuno (1978) Different arrangements of phagolysosome membranes which depend upon the particles phagocytized. Exp. Cell Res. 111, 437-449.

Savanat, T. & E. R. J. Pavillard (1964) The ability of Amoeba proteus to kill Salmonella entritidis introduced by microinjection and the influence of opsonins on intracellular killing. Aust. J. Exp. Biol. Med. Sci. 42, 615-624.

Sbarra, A., A.Jacobs, R.R.Strauss, B.B.Paul, & G.W.Mitchell (1971) The biochemical and antimicrobial activities of phagocytizing cells. Am. J. Clin. Nutr. 24, 272-281.

Sigma Technical Bullitins, Sigma Chemical Co.
No. 104 (1974) The colorimetric determination of phosphatase.
No. 670 (1974) The colorimetric determination of inorganic phosphorus.
No. 675 (1978) The colorimetric determination of 5'-nucleotidase.

Silverstein, S. C., R.M.Steinman & Z.A.Cohn (1977) Endocytosis. Ann. Rev. Biochem. 46, 669-722.

Smith, H. (1968) Biochemical challenge of microbial pathogenicity. Bact. Rev. 32, 164-184.

Smith, H (1977) Microbial surfaces in relation to pathogenicity. Bact. Rev. 41, 475-500.

Stockem, W. (1978) Fine structure of cytoplasmic crystals in Amoeba ptoteus. Cytobiologie 17, 301-306.

Stockem, W. & H. P. Klein (1979) Pinocytosis and locomotion in amoeba XV. Demonstration of Ca -binding sites during induced phagocytosis in Amoeba proteus. Protoplasma 100, 33-43.

Stokes, G. V. (1973) Formation and destruction of internal membranes in L cells infected with Chlamydia psittaci. Infect. Immun. 7, 173-177.

Stokes, G. V. (1974) Proteinase produced by Chlamydia psittaci in L cells. J. Bacteriol. 118, 616-620.

Taylor, F. J. R. (1976) Autogenous theories for the origin of eukaryotes. Taxon 25, 377-390.

Trager, W. (1974) Some aspects of intracellular parasitism: Intracellular protozoa enter host cells in subtle ways and escape the cell's digestive process. Science 183, 269-273.

Ulsamer, A. G., P.L.Wright, M.G.Wetzel & E.D.Korn (1971) Plasma and phagosomes of Acanthamoeba castellanii. J. Cell Biol. 51, 193-215.

Warren, L. & M. C. Glick (1971) The isolation of the surface membranes if animal cells: A survey. Biomembranes 1, 257-288.

Widnell, C. C. (1972) Cytochemical localization of 5'-nucleotidase in subcellular fractions isolated from rat liver. I. The origin of 5'-nucleotidase activity in microsomes. J. Cell Biol. 52, 542-558.

Wise, G. E. & C. J. Flickinger (1970a) Cytochemical staining of the golgi apparatus in Amoeba roteus. J. Cell Biol. 46, 620-626.

Wise, G. E. & C. J. Flickinger (1970b) Relation of the golgi apparatus to the cell coat in amoebae. Exp. Cell Res. 61, 13-23.

APPENDICES

APPENDIX I

Modified Chalkley's Medium

The concentrated stock solutions were prepared as follows and stored in a refrigerator (4 ℃).

Solution I. (x1000)

$NaCl$ - - - - - - - - - - - - - - - - -29.20 gm (0.5 mol)

KCl - - - - - - - - - - - - - - - - - 3.75 gm (0.05 mol)

$NaHCO_3$ - - - - - - - - - - - - - - - 0.84 gm (0.05 mol)

$Na_2HPO_4.7H_2O$ - - - - - - - - - - - - 1.90 gm (7.2 m mol)

Glas distilled water was filled to 1 liter.

Solution II (x2000)

$CaCl_2.2H_2O$ - - - - - - - - - - - - - 14.7 gm (0.10 mol)

Glass-distilled water was filled to 1 liter.

A working solution was prepared by adding 1 ml of Solution I and 0.5 ml of Solution II to a liter of glass-distilled water.

APPENDIX II

Culture Medium for *Tetrahymena*

1. Stock Solution of Salt and Vitamin Mixture

A. Salt Solution

K_2HPO_4 - - - - - 5.00 gm

$MgSO_4.7H_2O$ - - - 0.50 gm

$Zn(NO_3)_2.6H_2O$ - 0.25 gm

Thiamine.HCl - - 0.05 gm

Pyridoxyl.HCl - 0.10 gm

Sodium acetate 50.00 gm

$FeSO_4.7H_2O$ - - - 0.025 gm

$CuCl.2H_2O$ - - - 0.025 gm

Cholesterol - - 0.05 gm

Glass-distilled water to 1000 ml

B. Vitamin Solutioon

Riboflavin - - - - - 0.01 gm

Calcium pentothene - 0.01 gm

Niacin - - - - - - - 0.01 gm

Folic acid - - - - - 0.001 gm

Thioctic acid - - - - 0.001 gm

Glass distilled water to 50 ml

The stock solution of salt and vitamin mixture was prepared by adding 20 ml of Vitamin solution to 1000 ml of Salt solution. After dividing into aliquots of 10 ml each in polypropylene culture tubes, the aliquots were kept frozen in a freezer (-20 °C).

2. Culture Medium

Liver concentrate - - - - - - - - - 2 gm

Proteose peptone - - - - - - - - - - 20 gm

Salt and Vitamin Mixture - - - - - 10 ml

Glass D.W. was filled to 500 ml

The medium was divided in 250-ml flasks with 50 ml each, 500-ml flasks with 100 ml each, or 1000-ml flasks with 200 ml each, and autoclaved for 15 minutes at 121 °C, 15 atm.

VITA

Tae In Ahn was born in KyungBuk, Korea, on January 15, 1947. He attended an elementary school in AnDong and the secondary school in Seoul. He served for three years in the Korean Army while he was a college student and received his Bachelor of Science degree in Science Education from Seoul National University, Korea, in February 1974.

He enrolled in the Graduate School at the University of Tennessee, Knoxville, in September 1975. He received his Master of Science degree in Zoology in August 1977 and his Ph.D degree in Zoology in March, 1981, from the same University.

He has been happily married to the former Sang Sook Choi of Seoul Korea since August 1977, and they are the parents of a son, Dale born in May 1978.