SEPARATION OF OUTER AND CYTOPLASMIC MEMBRANES OF *MYXOCOCCUS XANTHUS*

by

WILKIN LUI
The University of Manitoba
Canada

A Thesis
submitted to

The Faculty of Graduate Studies and Research
THE UNIVERSITY OF MANITOBA

in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September, 1982

SEPARATION OF OUTER AND CYTOPLASMIC MEMBRANES OF *MYXOCOCCUS XANTHUS*

BY

WILKIN LUI

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

Four different methods are described for the isolation of the cytoplasmic and outer membranes of Myxococcus xanthus, three of which, used sucrose gradrent centrifugation to effect separation. The crude membrane preparations were separated into three fractions of different densities. The identity and the purity of each fraction was determined by assaying for outer and cytoplasmic membrane markers such as Succinate dehydrogenase activity; Nicotinamide adenine dinucleotide oxidase activity and lipopolysaccharides. In one method, the lighter fraction (ρ = 1.209) was found to be outer membrane, the intermediate fraction (ρ = 1.170) appeared to be hybrid membrane fraction which contained some enzyme activity and lipopolysaccharides. The heaviest fraction (ρ = 1.148) appeared to be the cytoplasmic membrane. In the other two methods, three fractions of similar densities were isolated. However, assay of the enzymatic and chemical markers showed that the lighter fraction was the cytoplasmic membrane while the denser fraction appeared to be outer membrane. The middle fraction in both cases contained significant amounts of enzyme activities and lipopolysaccharides. In all three methods, Sodium dodecylsulfate polyacrylamide gel electrophoresis resolved approximately 30 different polypeptides; however there was no significant difference in electropherograms of light and heavy fractions.

In the fourth method outer and cytoplasmic membranes were separated by differential solubilization of the crude

membrane fraction with Triton X-100. The lipopolysaccharides content and succinate dehydrogenase activity suggested that the Triton-insoluble fraction was outer membrane while the Triton-soluble fraction was cytoplasmic membrane. SDS polyacrylamide gel electrophoresis showed differences between the two factions.

In memory of my grandmother, Wai-Fong,

to my family,

and to Maxie.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. P. Y. Maeba, of the Department of Microbiology, University of Manitoba, for his support and guidance during this study.

I would like to thank Grant McCarty and Dominic Tsang for their helpful advice and friendship.

I also would like to thank Lilian Yu for helping me prepare this manuscript. Finally, a thanks to my parents for their support and encouragment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	- Absorbance
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease I
EDTA	- ethylenediaminetetraacetic acid
HEPES	- N-2-hydroxyethylpiperazine-N'-Z'-ethanesulfonic acid
KDO	- 2-Keto-3-deoxyoctonate
LPS	- lipopolysaccharide
RNA	- ribonucleic acid
RNase	- ribonuclease
rpm	- revolution per minute
SDS	- sodium dodecyl sulfate
TRIS	- tris (hydroxymethyl) aminomethane

INTRODUCTION

INTRODUCTION

The bacterium, Myxococcus xanthus, exhibits a developmental life cycle. When nutrients are depleted from solid medium, cells aggregate to form multicellular fruiting body. The possibility that cell surface components may be involved in the developmental process inspired a search for a simple method to separate and isolate the membrane of vegetative or developing cells so that their components could be studied.

The cell envelope of gram-negative bacteria, including Myxococcus xanthus, consists of two typical membranous structures; outer and cytoplasmic membranes. Miura and Mizushima (1969) first described separation of cell envelope of E. coli into two fractions, enriched respectively for outer and cytoplasmic membranes, by isopycnic sucrose density gradient centrifugation of membranes obtained by lysing spheroplasts. A totally different approach, developed by Schnaitman (1971 a) was based on the different solubility properties of outer and cytoplasmic membranes in nonionic-detergents such as, Triton X-100. These techniques led to a greater understanding of the structure and function of the outer membrane of gram-negative bacteria. However, most of the information has come from the analysis of the outer membranes of Enterobacteriaceae. Only one author has reported the separation of outer and cytoplasmic membrane of M. xanthus. The object of this research was to develop a method to isolate and characterize the membranes of M. xanthus.

HISTORICAL

HISTORICAL

Myxobacteria are a group of closely related chemoheterotrophic gram-negative bacteria with a complex life cycle. They were first described by Roland Thaxter in 1892 in a paper published in the Botanical Gazette as "... bright orange-colored growths occuring upon dead wood, fungi and similar substances ...." (Thaxter, 1892).

Myxobacteria are ubiquitous in nature occuring in soil and frequently develop on decomposing plant material, the bark of living trees or animal dung (McCurdy, 1974). They are distinguished from most other gram-native bacteria by their gliding motility, by their capacity to degrade microbial cells and by the formation of pigmented, flat, spreading colonies on solid substrates. They are distinquished from other gliding bacteria, such as Cytophagas and Flexibacteria, by the composition of their DNA which is 67 to 71 mole-percent in guanine and cytosine. They are unique among bacteria in their capacity for multicellular development (Kaiser, 1979).

Myxobacteria propagate by means of two interlocking cycles, one vegetative and the other developmental. When Myxobacteria grow vegetatively, multiplication is by binary tranverse fission. Under appropiate enviromental conditions, the cells enter a cycle of development in which cells aggregate to form fruiting bodies whose shapes are genus-specific. Some of the cells within fruiting bodies are individually transformed into resting cells called myxospores which are

resistant to heat, sonication and dessication. Myxospores can subsequently germinate to give vegetative cells under favourable conditions (Kaiser, Manoil and Dworkin, 1979).

The order, Myxobacterales, is subdivided into four families according to the morphology and structure of vegetative cells and myxospores, as well as the ability of some members to form spores in elaborate sporangia (McCurdy, 1974). The four families are: Myxococcaceae; Archangianceae; Cystobacteraceae and Polyangiaceae.

Myxococcus is the only genus in the family Myxococcaceae. Vegetative cells of Myxococcus are straight to slightly tapered. The myxospores produced are not enclosed in a sporangium and they are refractile and can be either spherical or ellipsoidal. The fruiting body formed by Myxococcus is a mound of cells covered by slime, slightly elevated from the surface (Wireman & Dworkin, 1975).

Myxococcus xanthus, the myxobacteria with which most of the recent work has been done, are slender, flexible rods, 0.5 - 1.0 by 4.0 - 10.0 um. They are capable of aerobic growth in a dipersed state in either complex or defined media. Structurely the vegetative cells of Myxococcus xanthus are not differ in any significant way from other gram-negative bacteria; although Johnson and White (1972) showed that the peptidoglycan did not exist as a contineous layer but in patches, connected by trypsin-SDS-sensitive material.

In nature, M. xanthus secretes bacteriolytic enzymes and proteases that permit it to feed on other bacteria (Sudo

& Dworkin, 1972). Dworkin (1962) developed a chemically defined medium which contained 17 amino acids and various salts that supported continuous growth of Myxococcus xanthus. No stimulation of growth was found with the addition of a variety of organic compounds, such as sugars; sodium acetate, sodium citrate, guanine and uracil, with the exception of glycogen. Bretscher (1977) later was able to cultivate M. xanthus in a defined liquid medium (A Medium) containing pyruvate and asparate as carbon and energy sources and various salts, leucine, isoleucine, valine, spermidine, asparagine and vitamin B_{12}. Growth was slow but continuous. When vitamin B_{12} was left out, methionine was required, suggesting that M. xanthus carried out a B_{12}-dependent methylation of homocysteine to methioine. Isoleucine, leucine and valine appeared to be essential amino acids because virtually no growth occured in their absence. Asparagine and spermidine, although not essential, gave a very slight stimulation of growth. M. xanthus can also be cultivated on complex medium (eg. 1% Difco Casitone and $MgSO_4$) the generation time is considerably shortened decreasing from 22 to 30 hours (Bretscher & Kaiser, 1977) in A Medium to 200 to 250 minutes in complex medium (Wireman & Dworkin, 1975).

If a suspension of M. xanthus is placed on agar medium on which they can grow and divide for a short time before nutrients become limiting, they shift from growth to development and begin to migrate, by gliding motility, to aggragation centers. Within one day translucent mounds of vegetative

cells arise at many sites. The mounds then become more defined and finally turn dark and opaque as the rod-shaped vegetative cells within them shorten into spherical, coated spores. Depending on the nutrient and cell density, mature fruiting body formation and conversion to myxospores requires 48 - 72 hours.

Working with three classes of fruiting-defective mutants. Kaiser, Manoil and Dworkin (1979) suggested that the shift from vegetative growth to fruiting body formation could be divided into three parts: initation by starvation, aggregation and fruiting body construction and finally myxospore formation.

Hemphill and Zahler (1968) using a defined medium, showed that amino acids served not only as substrate for protein synthesis but also as a source for carbon and energy. Bretscher & Kaiser (1977) further demonstrated that fruiting body formation was induced by limitation of any one of the essential amino acids or by starvation for carbon and energy or for inorganic phosphate. They suggested that fruiting could be induced directly by decreased levels of essential amino acids. Alternatively the starvation for carbon energy source or phosphate can indirectly induce fruiting by limiting endogenous synthesis of amino acid. Campos & Zusman (1975) and Yajko & Zusman (1978) detected an increase in the intracellular level of cAMP during early stage of fruiting. They suggested that this increase was in response to starvation and that cAMP stimulated the expression of fruiting-specific

genes thus initating fruiting development in M. xanthus.

Wireman and Dworkin (1975) suggested the requirement for diffusible chemical signals between cells to coordinate developmental processes. Wireman & Dworkin (1976, 1977) observed massive lysis of myxobacterial cells accompanied fruiting body formation. Lysis typically claimed 80% of the vegetative rods, the remaining 20% differentiating into spores within fruiting bodies. The commitment of a cell to proceed towards lysis or myxospores conversion is not understood. The products of lysed cells may provide components for fruiting body construction and myxospore formation.

When the formation of the fruiting body is complete, cells within them start to convert to myxospores. Each fruit can consist of 40,000 or more myxospores (D. White, 1975). Until recently, the conversion was studied with log phase liquid cultures which were induced to form myxospores by addition of glycerol to 0.5M (Sadler & Dworkin, 1966). This is a nonphysiological, but highly relevant, process in which myxospores are induced without prior aggregation. Other compounds including dimethyl sulfoxide or those with primary or secondary alcohol groups, such as phenethyl alcohol, propanol, ethylene glycol and butanol, can replace glycerol (Sadler & Dworkin, 1966). The mechanism by which these reagents induce myxospore formation is not known.

Within 0.75 hour after the addition of glycerol the rod-shaped cells start to shortern. By one hour the cells become ovoid then begin to turn into spheres. It takes eight hours

for a cell to convert both biochemically and physiologically to myxospore (White, 1975). The myxospores formed by glycerol induction resemble those formed within fruiting body in gross morphology and resistance properties (krottel et al, 1975).

During myxospore induction in M. xanthus there are substantial changes: (i) the structural conversion of cell, (ii) conversion to a state of metabolic quiescence, and (iii) preparation for subsequent germination (Wireman & Dworkin, 1975).

Metabolic studies have shown that there are changes in a number of enzyme activities associated with glycerol-induced myxospore formation. As in Bacillus endospore formation, certain enzyme activities decrease at specific stages of myxospore formation; eg., aspartokinase activity drops 80% in the first hour. At the same time, several other enzyme activities increase, including those of glyoxylate cycle (Dworkin, 1972), isocitrate lyase and malate synthase, as well as fructose-1-6-diphosphatase and six enzymes responsible for the synthesis of UDP-N-acetylgalactosamine from fructose-6-phosphate (White, 1975). Furthermore, the rate of oxygen uptake is reduced by about 80%.

The peptidoglycan content in vegetative cells and myxospores are similar; however, White et al (1968) and Johnson et al (1972) found that the amount of peptide crosslinkage between glycan chains was significantly higher in myxospores than in vegetative cells and suggested that this contributed to the increase structural resistance of the myxospores. In

addition, the myxospore peptidoglycan appears to contain covalently bound glucose.

Rosenberg et al (1967) found that during myxospore formation DNA synthesis continues until existing rounds of replication are completed; no new rounds are initiated. Net RNA synthesis ceases immediately although increased turnover takes place. Foster and Parish (1973) have also found differences in the physical properties and in the protein subunit composition of the 30S subunit of the myxospore ribosome. Net protein synthesis continues during the morphological conversion, but after two hours, net protein synthesis ceases, although a high rate of protein turnover continues (Orlowski and White 1974).

A layer of spore coat appears together with the formation of myxospores. Kottel et al (1975) analysed the content of the coat and found that it consists mainly of polysaccharide (75% by weight) with protein (14%) and glycine (8%). The polysaccharide component consists predominantly of glucose and galactosamine.

Even though there are many similarities between the glycerol-induced myxospore and the fruiting body myxospore there are structural and physiological differences between them. Only fruiting body spores contain protein S as an outermost layer on surface of spores (Inouye et al, 1979). There are also differences in germination properties and metabolic rate (Kaiser et al, 1979). Morrison & Zusman (1979) working with mutants of M. xanthus that are temperature

sensitive for fruiting-body formation, proposed that there are two different pathways operating during development: a sporulation pathway and a aggregation and fruiting body formation pathway which are independent of each other.

Outer Membrane of Gram-negative Bacteria

E. coli, a gram-negative bacterium, is surrounded by five electron dense tracks in contrast to the typical double-tracked layer of a unit membrane (Murray et al, 1965). Based on the fact that the central track disappeared after lysozyme treatment of cells, it was concluded that the cell wall or the peptidoglycan layers exists between the two double-track layers, which represent two distinct membrane systems. Both appear as typical unit membrances, having a thickness of about 75Å. These membrances are called the outer membrane and the inner, or cytoplasmic membrane.

Although outer membranes of gram-negative bacteria are morpholographically similar to cytoplasmic membranes, they contain less phospholipid, fewer proteins and a unique carbohydrate component, lipopolysaccharide (LPS). Functionally the outer membrane is quite distinct: it acts as a diffusion barrier against various compounds, for example antibiotics; it contains receptors for bacteriophages and colicins; it is involved in the process of conjugation and cell division; it contains specific uptake systems for nutrients, such as iron, vitamins and carbohydrates; and it contains non-specific passive diffusion pores that allow the passage of low molecular weight substrates. The outer membrane also provides a

protective enviroment for enzymes that reside in the periplasmic region, the area between the cytoplasmic and outer membrane. In addition, it plays a role in maintaining the structural integrity of the cell.

Ultrastructual and biochemical data suggest that the organization of outer membrane component is highly asymmetric. The work of Smit & Nikaido (1975) and Kamio & Nikaido (1976), showed by freeze-fracture electron microscopy and electron-spin resonance that in wild type Salmonella typhimurium the phospholipid were localized such that most of the phospholipid molecules were localized in the inner half of outer membrane. On the other hand, the outer half of the outer membrane was comprised mainly of lipopolysaccharide. In S. typhimurium, Smit et al (1975) calculated that there were approximately 2.5×10^6 molecules of lipopolysaccharide per cell localized exclusively in the outer leaflet of the outer membrane. They occupied about 45% of the surface of the outer membrane. The domains occupied by LPS were distinct from those occupied by phospholipids. Kaurn & Nikaido (1967) used CNBr-activated dextran as a non-penetrating agent, to label amino groups of exposed outer membrane proteins of S. typhimurium mutants with defective LPS. The short polysaccharide chains associated with the LPS allowed the reagent access to the outer membrane surface. They found that most of the proteins in the outer membrane could be labelled and were exposed to the external medium. The organization of outer membrane is complicated by the fact that peptiodglycan is closely associated with

the outer membrane (Mirelman, 1979), some outer membrane proteins specifically interacting with peptidoglycan.

The phospholipids in the total membrane of E. coli are phosphatidylethanol amine , phosphatidylglycerol and cardiolipin. All have similar fatty acid compositions consisting of palmitic, myristic palmitoleic and cis-vaccenic acids (Cronan, 1979). Osborn (1972) reported that the outer membrane had a higher proportion of phosphatidylethanolamine than did the inner membrane and was thus deficient in phosphatidylglycerol and cardiolipin. Although phospholipids are primarily localized in the inner half of the outer membrane, it is not certain whether some may be present as phospholipid bilayers. Kamio & Nikaido (1976) and Smit et al (1975) showed that in wild type S. typhimurium the phospholipids were present as monolayers. However in mutants with decreased levels of LPS, phospholipids were detected in the outer layer, suggesting a substitution of phospholipid for LPS. This was supported by the work of Bayer (1975). Examination of electron micrographs of freeze-fractured cells suggested that outer membrane did not have a typical lipid bilayer structure. On the contrary, Van Alphen et al (1977) repeated analogous experiments using various E. coli mutants and opposed Nikaido's interperation in favour of the bilayer proporsal. Cronan (1979) suggested a model in which phospholipid bilayer occurs only in small patches.

Lipopolysaccharide is located on the outer surface of outer membrane. Bacterial LPS can be roughly divided into

three parts: Lipid A, core polysaccharide and O-antigen polysaccharide chain. Lipid A of LPS is believed to be associated with the hydrophobic zone of outer membrane while the hydrophilic polysaccharide protrudes from the outer membrane.

Kamio and Nikaido (1977) showed that most of the protein in the outer membrane were exposed to the external medium. This is quite conceivable because most outer membrane proteins serve as receptors for various nutrients, phages and colicins. A striking feature that one protein can serve as a receptor for several structurally unreleated substrates (Braun and Hantke, 1977). However this does not necessarily mean that they function in the same way for each substrate.

Proteins in outer membrane are classified into major and minor proteins. The definition of major proteins is rather arbitrary. A minor protein may become a major proteins when its production is fully induced (Nikaido, 1979).

The outer membrane of E. coli K-12 contains at least three major protein; matrix protein, ompA protein (tol G protein) and lipoprotein. Matrix proteins were characterized by their tight but noncovalent association with peptidoglycan. Two matrix proteins, Ia and Ib are present in E. coli K-12. They were purified from cultures grown under conditions allowing formation of either Ia or Ib or from mutants lacking one of the two proteins (DiRienzo et al, 1978). Nakae (1976) suggested that the matrix proteins form passive diffusion pores so that the proteins are also called "Porin". A striking feature of matrix protein was their extremely high content

of β-structure, in contrast to many other "intrinsic" membrane proteins, which show high content of α helix (Rosenbush, 1974; Nakanura & Mizushima 1976).

OmpA protein or tol G protein exists in large quantities in the outer membrane. The molecular weight is approximately 30,000 and like the porins, contained a high β-structure content (Nakanura & Mizushima, 1976). Tol G protein was susceptible to partial proteolytic digestion suggested that part of this protein was exposed to the outer surface supporting the fact that it serves as a receptor for certain phages (Manning et al, 1976). Another function of the tol G protein is that it is required for F pilus-mediated conjugation.

Lipoprotein is the most abundant protein in the cell in terms of number of molecules and has many unique features. Braun and Rehn (1969) first reported that the lipoprotein was covalently linked to peptidoglycan. Inouye et al (1972) later showed that the lipoprotein was also present in the E. coli membrane without covalent linkage to the peptidoglycan. Although the exact function of the lipoprotein is still obscure, analysis of lipoprotein mutants has revealed that the lipoproteins play an important role in maintaining the integrity of the outer membrane structure (DiRienzo et al, 1978).

There are about 10-20 minor proteins present in the outer membrane. Many minor proteins have been identified as receptors for phages and colicins. Most of them, as well as additional proteins with no known receptor functions, are now

known to have vital roles in the transport of nutrients through the outer membrane. James (1975) also suggested that a minor protein G has a role in the coordination of DNA replication and cell elongation.

Detailed studies of gram-negative cell walls require the isolation of cell walls uncontaminated by cytoplasmic membrane. The first preparation of "pure" outer membrane was obtained, rather unexpectedly by Bishop and Work (1964) and later by Rothfield and Pearlman-Kothenez (1969). They purified vesicles formed by the pinching off of protrusions of outer membrane that arose during unbalanced growth.

The first successful physical separation of outer membrane from the cytoplasmic membrane was described by Miura and Mizushima (1969). Their method included osmotic lysis of EDTA-Lysozyme induced spheroplasts of E. coli, followed by extensive dialysis of the cell envelope fraction against EDTA and equilibrium centrifugation of this fraction in a sucrose density gradient. Two bands were obtained from the centrifugation. The lighter band contained lipoprotein and had a "unit membrane" structure. Relatively large amounts of ATPase and components of the electron-transfer system were also detected. This fraction presumably corresponded to the cytoplasmic membrane. The denser fraction contained a large amounts of carbohydrate and NADH dehydrogenase and lacked respiratory compoments. Furthermore immunological studies showed this fraction was located on the outside of the cell surface. The carbohydrate content of this fraction

was only about 5%, on contrast to 50% reported by Rothfield et al (1969), and represented an extensive loss of LPS during purification, probably due to the use of EDTA. Regardless this method for membrane separation was employed with minor modification by other workers.

Osborn (1972) used sonication to lyse S. typhimurium spheroplasts before sucrose gradient centrifugation. The advantages were that only one gradient centrifugation was required and this method could be applied to a variety of bacterial strains. The concentration of lysozyme was important. When the concentration was too low, spheroplast formation was incomplete. If too high a concentration resulted in agglutination of cells and spheroplasts of poor quality. With the right concentration of EDTA loss of LPS could be avoided.

A different method was devised by Schnaitman (1970)[b], in which cells were broken by passage through a French pressure cell. This method has two distinct advantages over that requiring spheroplasts. First, the morphological featurs of the cell wall were preserved during isolation, permitting its identification. Secondly, it was possible to study the incorporation of components into the cell wall which might otherwise be lost or altered during spheroplast formation.

Another approach for purifying the outer membrane is based on the solubility properties of the different membranes in detergents. DePamphilis and Alder (1971) and Schnaitman (1971, a, b) found that outer membranes were not solubilized

in 1.5 - 2.0% Trition X-100, a nonionic detergent, provided Mg^{2+} was present although cytoplasmic membranes were completely dissociated. Unfortunately, about one-half of the LPS and two-thirds of the phospholipids of the outer membrane were lost. In contrast, there was no significant loss of protein.

Still another method for the isolation of outer membrane involved the direct fractionation of crude cell envelope by a particle electrophoresis apparatus (White et al, 1972).

With improved methods for separation of outer and cytoplasmic membranes alternation in the outer membrane could be analyzed. Mizuno and Kageyama (1978) studied several strains of Pseudomonas aeruginosa and found the outer membrane protein composition was altered by the cultivation medium. For instance, a few outer membrane protein of P. aeruginosa were induced in an iron-limited medium. Similarly, it was reported that certain outer membrane proteins of E. coli of high molecular weight (74,000-81,000) were induced under iron-limited conditions. Koplow and Goldfine (1974) found marked changes in the relative amount of protein, phospholipid and lipid A in the outer membrane fraction among various LPS mutants of E. coli. Schnaitman (1974) suggested that the major outer membrane proteins of E. coli, Shigella sp. and Salmonella typhimurium vary considerably due to strain and cultural differences.

It is possible that outer membrane components may be specifically involved in the development process in M. xauthus. In particular, the components may provide receptors

for chemotactic substances, may be involved in gliding motility, may play a role in physical interaction between cells or provide means of communication between cells.

The current study was to develop a method for isolating outer and cytoplasmic membrane and to observe changes in the outer membrane during various stage of fruiting body development.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism

Four strains of Myxococcus xanthus, A^+S^+ (wild type), A^+S^-, A^-S^+ and A^-S^- were obtained from D. Kaiser (Stanford University, California). The organism was routinely grown in 1% (w/v) Difco casitone containing 0.008M $MgSO_4$ (CM medium) at 30°C with vigorous shaking in a Model G-76 Gyrotory water bath shaker (New Brunswick Scientific Company) to provide aeration. For storage, log phase culture were made 0.5M with sterile glycerol, distributed in 1.5 ml volume into sterile glass vials, then stored at -70°C.

Growth

Stock cultures were inoculated into 40 ml of CM medium in 250 ml Erlenmyer flasks and grown to mid-log phase as described above. Ten ml of this culture was inoculated into 400 ml of CM medium in 2 liter Erlenmyer flasks which were shaken at 160 rpm in a Model G-53 Gyrotory shaker (new Brunswick Scientific Company). In this case, incubation was carried out at 28°C, the temperature at which the incubation room containing the shaking apparatus was maintained. When the culture density reached 200 Klett units (Red filter), the cells were harvested by centrifuged at 4°C in a RC-5 Superspeed refrigerated centrifuge (Sorvall Inc.) with a Sorvall type G-53 rotor at 9,000 rpm for 20 minutes. Approximately 4 g (wet weight) of cells were obtained from 400 ml of medium. For storage, harvested log phase cells were washed once in

50 mM Tris buffer pH 7.8 and then stored at -70°C.

To obtain cells in the developmental cycle, i.e., during fruiting bodies formation, the centrifuged cells were resuspended in modified A-1 minimal medium (see later) to a density of approximately 3.0×10^{10} cells per ml and spotted on the same medium solidified with 1% agarose. The cell suspension was transferred via a peristaltic pump, allowing 10-20 ul spots to form on the solid surface of the medium. Plates were incubated at 30°C to allow development.

For fruiting body formation, minimal medium A-1 of Bretscher and Kaiser (1978) was modified by the omission of asparagine, phenylalanine, and spermidine. The final composition of the modified medium in 1 liter of distilled water was as follows:

Agarose	10.00 g
L - isoleucine	0.10 g
L - leucine	0.05 g
L - methionine	0.01 g
L - valine	0.10 g
Sodium pyruvate	5.00 g
Potassium aspartate	5.00 g
Vitamin B-12	1.00 mg
$MgSO_4$	0.96 g
$CaCl_2$	1.10 mg
$FeCl_3$	1.60 mg
$(NH_4)_2SO_4$	0.50 mg

Tris-HCl, pH 7.6	0.01 M
KH_2PO_4-K_2HPO_4, pH 7.6	0.01 M

The medium was sterilized in an autoclave for 20 minutes at 121°C. Vitamin B-12, sterilized by Millipore filtration, was added when the medium had cooled to 50°C. The medium, allowed more or less synchronous formation of fruiting bodies.

Preparation of Crude Membrane Fractions

Total membranes were prepared by using three different methods.

Method I

This was based on the method used by Schnaitman (1970)[b]. Twenty-five grams (wet weight) of harvested log phase cells were washed once in 50 mM Tris buffer pH 7.8 and then resuspended in equal volume of 50 mM Tris buffer containing 1 mM EDTA (w/v). The suspension was homogenized twice for 1 minute in a Sorvall omni-mixer set for the highest speed in order to remove capsular material. The cells were again centrifuged at 13,200 x g for 10 min. in a RC2-B superspeed refrigerated centrifuge (Sorvall Inc.) and resuspended in 35 ml Tris-EDTA buffer. One milligram each of DNase I and Pancreatic ribonuclease A and the suspension was passed through a French pressure cell (Aminco) twice at 16,000 lb/in^2. $MgCl_2$ was added to the broken cell suspension to a concentration of 2 mM. Unbroken cells were removed by centrifugation at 20,200 x g for 15 min. The extract was then centrifuged for 90 minutes at 45,000 rpm in a Spinco 60 Ti rotor (Beckman Instrument

Company) at 5°C in an L2-65B Beckman ultracentrifuge. The pellet was suspended in 10 mM HEPES buffer, pH 7.4 and was referred to as total membrane.

Method II

This method, with minor alterations, was based on that of Irschik and Reichenback (1978).

Ten grams of harvested cells were washed once in 50 mM Tris buffer pH 7.8 and suspended in 30 ml of the same buffer containing 6.0M glycerol and 6 mg lysozyme. After incubation at room temperature for 15 min., the suspension was injected with a syringe fitted with a 25-gauge needle into 130 ml of ice cold Tris buffer containing 40 ug/ml each of DNase and RNase. The suspension was incubated at 5°C for fifteen minutes at which time 90% of the cells had become spheroplasts. The suspension was shaken on a water bath at 12°C and 0.3% Brij 35 and 1.35 mM EDTA were added. Upon this the suspension became quickly transparent. The mixture was held in an ice bath for 20 min. and then subjected to 10 sec. exposure to sonication at a setting of 2 on a Model 1000 Insonator (Ultrasonic System Inc.) fitted with a microtip. Cells and cell debris were removed from the homogenized lysate by centrifugation at 13,200 x g for 10 min. in a RC2-B superspeed refrigerated centrifuge (Sorvall Inc.). The supernatant was then centrifuged at 35,000 rpm for 90 min. in a 60 Ti rotor (Beckman Instrument Co.) at 5°C in a L2-65B Beckman ultracentrifuge. The pellet was washed then resuspended in 0.01M Tris-HCl buffer, pH 7.6, containing 0.01% Brij 35 and 1.35 mM EDTA

The suspension was the crude total membrane.

Method III

This method is based on the method of Irschik and Reichenbach (1978) as modified by Mizuno & Kageyama (1978).

Thirteen grams (wet weight) of cells were washed once in 50 mM Tris buffer, pH 7.8, and resuspended in 40 ml Tris buffer containing 6.0M glycerol and 9 mg lysozyme. After incubation at room temperature for 15 min., the suspension was injected with a syringe fitted with an 25-gauge needle into 200 ml of ice-cold Tris buffer, pH 7.8, containing 10 mg each of DNase and RNase and incubated at 5°C. About 90% of the cells became spheroplasts within 15 min. The suspension was then centrifuged at 20,200 x g for 15 min. to sediment the spheroplasts. Crude outer membranes were recovered from the supernatant by centrifugation at 96,000 x g for 60 min. The spheroplasts were resuspended in 60 ml of 0.5 mM Tris buffer, pH 7.8. It was then passed through a French pressure cell (Aminco) twice at 16,000 lb/in^2. Unbroken spheroplasts or cells was removed by centrifugation at 6,000 x g for 7.5 min. The supernatant was centrifuged at 150,000 x g for 90 min. The pellet, resuspended in 0.1M Tris buffer, pH 7.8, was designated as crude inner membrane fraction.

Separation of Outer and Cytoplasmic Membranes

The outer and cytoplasmic membrane fractions in the crude membrane fractions as well as the crude inner and outer were purified by isopycnic sucrose density gradient centrifugation.

The membrane fractions suspended in a small volume of distilled water, were layered onto discontineous sucrose gradients and centrifuged at 25,000 rpm for 20 h. in a Beckman SW 27 rotor. Fractions were collected by piercing the bottom of the tube and emptying contents with a peristaltic pump. The absorbance of each fraction was measured at 280 and 260 nm with Model 2000 spectrophotometer (Gilford). The refractive index of each fraction was determined with a Bausch and Lombe refractometer. These were converted to densities using the appropriate conversion tables. Peak fractions were pooled, diluted approximately two folds with a solution containing 0.1 mM $MgCl_2$, 0.25M sucrose, 0.01M NaCl and 0.02M phosphate buffer (pH 6.8). Particulate material was recovered by centrifugation in a 60 Ti rotor for 1 hour at 45,000 rpm. The samples were suspended in a small volume of 0.01 HEPES. buffer, pH 7.4, and dialized overnight against distilled water.

Separation of Outer and Cytoplasmic Membranes by Triton X-100

Method IV

This method of Schnaitman's (1971 a, b; 1975) was carried out by extracting total membrane with Triton X-100.

Cells were fractionated by French pressure cell as previously described (Method I). A suspension of crude total membrane fraction (1-10 mg protien/ml) was incubated in 2% Triton X-100 in 10 mM HEPES. buffer (pH 7.4) containing 10 mM $MgCl_2$ for 20 minutes at 23°C. The suspension was chilled and centrifuged in a Beckman 50 Ti rotor for 1 hour at

50,000 rpm to sediment the Triton - insoluble material. The supernatant fraction was placed on ice, and 2 volumes of ice-cold absolute ethanol were added with stirring. The sample was then allowed to stand overnight in a freezer and the protein precipitate, free of Triton X-100, was collected by centrifugation at 25,000 rpm for 1 hour in a Beckman 60 Ti rotor. Both the Triton - soluble and Triton - insoluble material were resupended in water.

Protein Assay

Protein content was determined by the method of Lowry et al (1951) with bovine serum albumin (fraction V, Sigma Chemical Co.) as standard.

Enzyme Assay

NADH oxidase: NADH oxidase was assayed as described by Osborn (1972). Incubation mixtures contained 50 mM Tris HCl, pH 7.5, 0.12 mM NADH, 0.2 mM dithiothreitol, and the membrane fraction (20 to 100 ug of protein) in a volume of 1.0 ml. The rate of decrease in absorbance at 340 nM was measured at 22°C in a Gilford 2000 recording spectrophotometer. Cyanide sensitivity was measured in reaction mixture containing 10 mM NaCN.

Succinate Dehydrogenase: succinate Dehydrogenase activity was assayed according to Osborn (1972). Incubation mixtures containing 60 mM phosphate buffer, pH 7.5, 10 mM NaCN, 30 ug of 2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) monotetrazolium bromide, 10 ug phenazine methosulfate, 25 mM succinate and

membrane fraction (5 to 100 ug of protein) in a volume of 1.0 ml. Absorbance at 550 nM was determined over a 10-min. period at 22°C in a Gilford 2000 recording spectrophotometer and the observed rates of reaction were corrected for blanks lacking succinate.

Lipopolysaccharide (LPS) Content

This was estimated by determination of KDO content (Osborn 1972) or by direct determination of Lipopolysaccharide as described by Janda & Work (1971).

KDO Assay: Membrane fractions (0.2 to 2 mg of protein) were precipitated with ice-cold 10% trichloracetic acid (5ml), collected by centrifugation at 4°C for 10 min. at 20,000 x g and washed twice with 5 ml of distilled water. The precipitate was suspended in 0.7 ml of 0.018 N H_2SO_4 and hydrolyzed at 100°C for 20 minute to liberate KDO from LPS. KDO was determined directly on the hydrolysate by the thiobarbituric acid method of Weissbach & Hurwitz (1959) with minor modifications. Hydrolyzed sample was added to 0.25 ml of 10 u moles of HIO_4 in 0.125 N H_2SO_4. After 20 min. at room temperature 0.5 ml of 100 u mole $NaAsO_2$ in 0.5 N HCl was added with shaking and the solution was permitted to stand for 2 min. Two ml of 0.3% thiobarbituric acid (pH .2) was added and the mixture was heated at 100°C for 20 min. when the mixture had cooled, the chromogen was extracted in 1.5 ml of Cyclohexanone and the absorbance was measured at 548 nm with a Gilford 2000 spectrophotometer.

Carbocyanide Method (Janda & Work, 1971)

To make the dye reagent, 10 mg of carbocyanine dye, 1-Ethyl-2-[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2 methylpropenyl] naphtho [1,2d] thiazolium Bromide, was dissolved in 20 ml of a mixture of equal parts of 1.4-dioxan and 0.03M sodium acetate buffer pH 4.05. A further 80 ml of sodium acetate buffer followed by 2 ml of 0.1 ml ascorbic acid was added. Membrane samples (containing 0.5 -1.0 ug LPS) mixed with 0.2 ml sodium acetate buffer and 0.3 ml of dye reagent and incubated in the dark for 5 - 10 min. The absorbance was measured at 472 nm with a Gilford 2000 spectrophotometer. LPS from E. coli (Sigma) was used as standard.

Absorbance spectra of some of the chromogens formed in the above colour assays were taken in a Shimadzu spectrophotometer MPS-50L.

Extraction of Lipopolysaccharide from M. xanthus

Bacteria (5 g dry weight) were suspended in 50 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA and 0.05% (w/v) sodium azide with a Sorvall omnimixer at top speed for 1 min. Lysozyme (0.1 g) was added and the suspension was stirred at $4^{o}C$ for 16 hr., then at $37^{o}C$ for 20 min. The mixture was blended at top speed in an omnimixer for 3 min. The volume of the suspension was adjusted to 100 ml with 20 mM $MgCl_2$ and RNase and DNase were added to final concentration of 1 ug/ml each. The suspension was incubated for 10 min. at $37^{o}C$ then 10 min. at $60^{o}C$, and blended again for 3 min. before phenol extraction.

The temperature of the suspension was raised to 70°C and an equal volume of 90% (w/v) phenol (preheated to 70°C) was added. The resultant mixture was stirred for 15 min. then rapidly cooled to 15°C by stirring in an ice water bath. Centrifugation at 18,000 x g for 15 min. permitted sharp definition of aqueous, phenol, and interfacial layers.

Aqueous and phenol phases were carefully removed by aspiration, the aqueous phase was retained while the phenol phase was discarded. The sediment and interfacial material remaining after removal of aqueous and phenol phases were resuspended in water and the extraction repeated. Pooled aqueous phases were dialyzed against water until no detectable phenol odour remained and were lyophilized. The lyophilized LPS was suspended in distilled water at concentration of 25 - 35 mg/ml and centrifuged at 1,100 x g for 5 min. The sedimeat was discarded and the supernatant fraction was centrifuged at 48,250 x g for 20 min. and the resulting pellet was assayed for LPS.

SDS Polyacrylamide Gel Electrophoresis

SDS acrylamide slab gels were prepared as described by Laemmli (1970). The stacking gel contained 3% acrylamide, 0.08% N, N'-bismetlylene acrylamide; 0.125M Tris-HCl (pH6.8) and 0.1% SDS. The separation gel contained 12.9% acrylamide; 0.35% bisacrylamide; 0.375M Tris-HCl (pH 8.8) and 0.1% SDS. Both the separation and upper stacking gels were polymerized by the addition of 0.025% by volume of tetramethylethylenediamine (TEMED) and ammonium persulfate. The electrode buffer (pH 8.3) contained 0.025M Tris-HCl and 0.192M glycine and 0.1%

SDS. Proteins in the membrane preparations were solubilized at 100°C for 1.5 min. in a solution which contained 0.0625M Tris-HCl (pH 6.8), 2.0% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue. Electrophoresis was carried out with a current of 30 mA per slab gel until the bromophenol blue marker reached the bottom of the gel (about 10 hr).

Proteins in the gel were fixed, stained and destained according to Fairbanks et al (1971) with slight modification. The solution used at each of the stage were: 1) 25% isopropyl alcohol, 10% acetic acid, 0.03% coomassie blue; overnight; 2) 10% isopropyl alcohol, 10% acetic acid, 0.003% coomassie blue; 6 - 9 hr and 3) 10% acetic acid, until the backgrond of the gel became clear.

Materials

Most of the reagents and chemicals were obtained from Fisher Scientific Company. β-Nicotinamide adenine dinucleotide, 2-thiobarbituric acid, phenazine methosulfate, [3-(4,5-Dimethyl thiazoly-2) 2,5-diphenyl tetrazolium bromide], sodium M-periodate, DL-dithiothertol, lysozyme (Egg white), deoxyribonuclease I (from Bovine pancreas) lipopolysaccharide (from E. coli) and EDTA were obtained from Sigma Company. 1-Ethyl-2-[3-(1-ethylnaphtho [1,2d]-thiazolin-2-ylidene)-2 methylpropenyl] naphtho [1,2d] thiozolium bromide, bis-acrylamide, acrylamide and N, N, N', N' tetramethylethylenediamine were purchased from Eastman. Brij 35 (30% solution) was obtained from Pierce. Ribonuclease A (Beef Pancreas in 0.1% phenol solution) was from ICN Pharmaceutial Inc.).

All chemicals were reagent grade or better.

RESULT

RESULT

Isolation of Membrane Fractions

Log phase cells were routinely used for membrane preparations. Cells that had been stored in -70°C up to three weeks yielded membranes that were indistinguishable from those of freshly harvested cells.

Method I

Total membranes of various strains of M. xanthus were prepared as described in Materials and Methods from cells disintegrated in the French press. Subsequent separation of outer and cytoplasmic membranes was achieved by sucrose density gradient centrifugation. The protein profiles of typical separation of membranes from the A^+S^+, A^+S^-, A^-S^+, and A^-S^- strains are shown in Figures 1 to 4, respectively. In all cases, two yellowish-brown bands were visible with the upper band (Fraction III) being thicker with less distinct edges than the middle band (Fraction II). Fraction with high A_{280} readings were pooled as indicated in the Figures and were used for enzyme and chemical assays. Although Fractions II and III were not well separated, they banded at the same positions when the pooled fractions were re-centrifuged in sucrose gradients. The profile in Figure 5 shows that Fractions I and III from A^+S^+ membranes were quite homogeneous while Fraction II might represent an intermediate fraction that contained unseparated Fraction I and II or their hybrids. Similar results were obtained when pooled fractions of A^+S^-, A^-S^+ and A^-S^- membranes were re-run.

Figure 1

Protein profile of total membrane of M. xanthus A^+S^+ obtained by sucrose gradient centrifugation formed with 3 ml of 60% sucrose, 6 ml of 50%, 6 ml of 45%, 6 ml of 40%, 6 ml of 35%, 6 ml of 30% and 2 ml of membrane sample. Fractions (39 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm. Fraction I, II and III were obtained by pooling the fractions indicated by the bars.

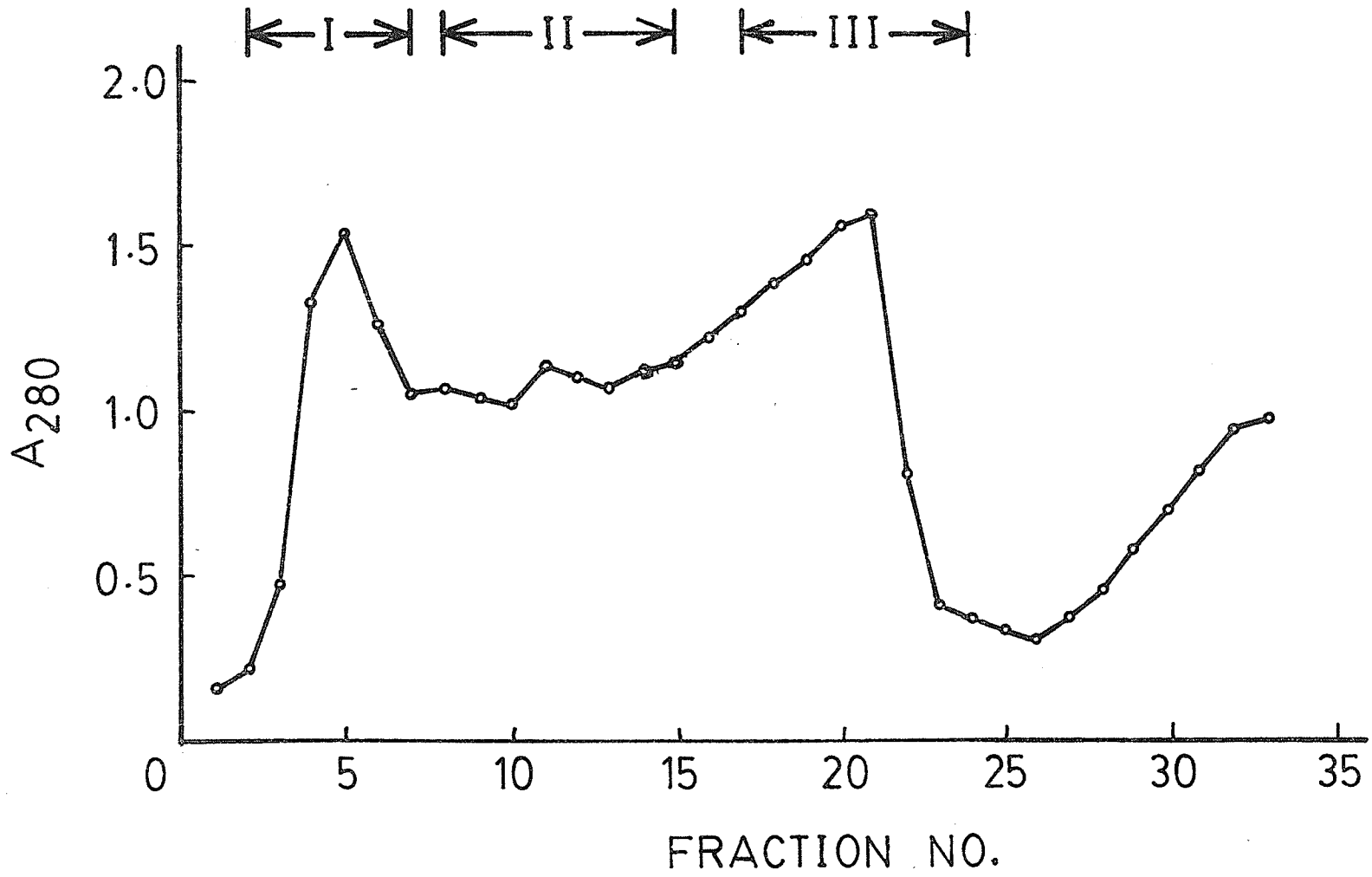

I
II
III
2·0
1·5
1·0
0·5
A_{280}
0
5
10
15
20
25
30
35
FRACTION NO.

Figure 2

Protein profile of total membrane of M. xanthus A^+S^- obtained by sucrose gradient centrifugation formed with 3 ml of 60% sucrose, 6 ml of 50%, 6 ml of 45%, 6 ml of 40%, 6 ml of 35%, 6 ml of 30% and 2 ml of membrane sample. Fractions (39 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm. Fraction I, II and III were obtained by pooling the fractions indicated by the bars.

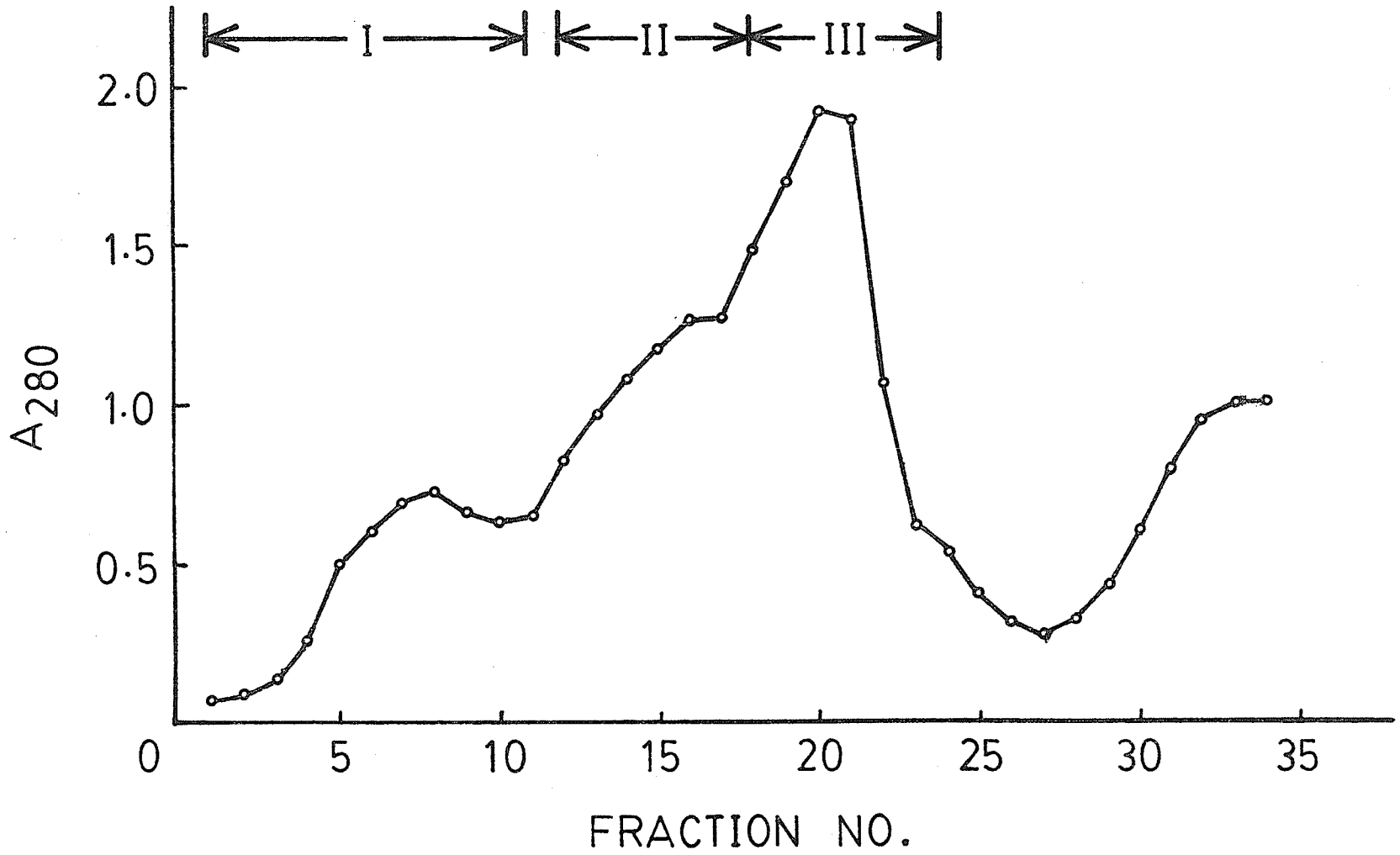

I
II
III
2·0
1·5
1·0
0·5
A_{280}
0
5
10
15
20
25
30
35
FRACTION NO.

Figure 3

Protein profile of total membrane of M. xanthus $A^{-}S^{+}$ obtained by sucrose gradient centrifugation formed with 3 ml of 60% sucrose, 6 ml of 50%, 6 ml of 45%, 6 ml of 40%, 6 ml of 35%, 6 ml of 30% and 2 ml of membrane sample. Fractions (39 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm. Fraction I, II and III were obtained by pooling the fractions indicated by the bars.

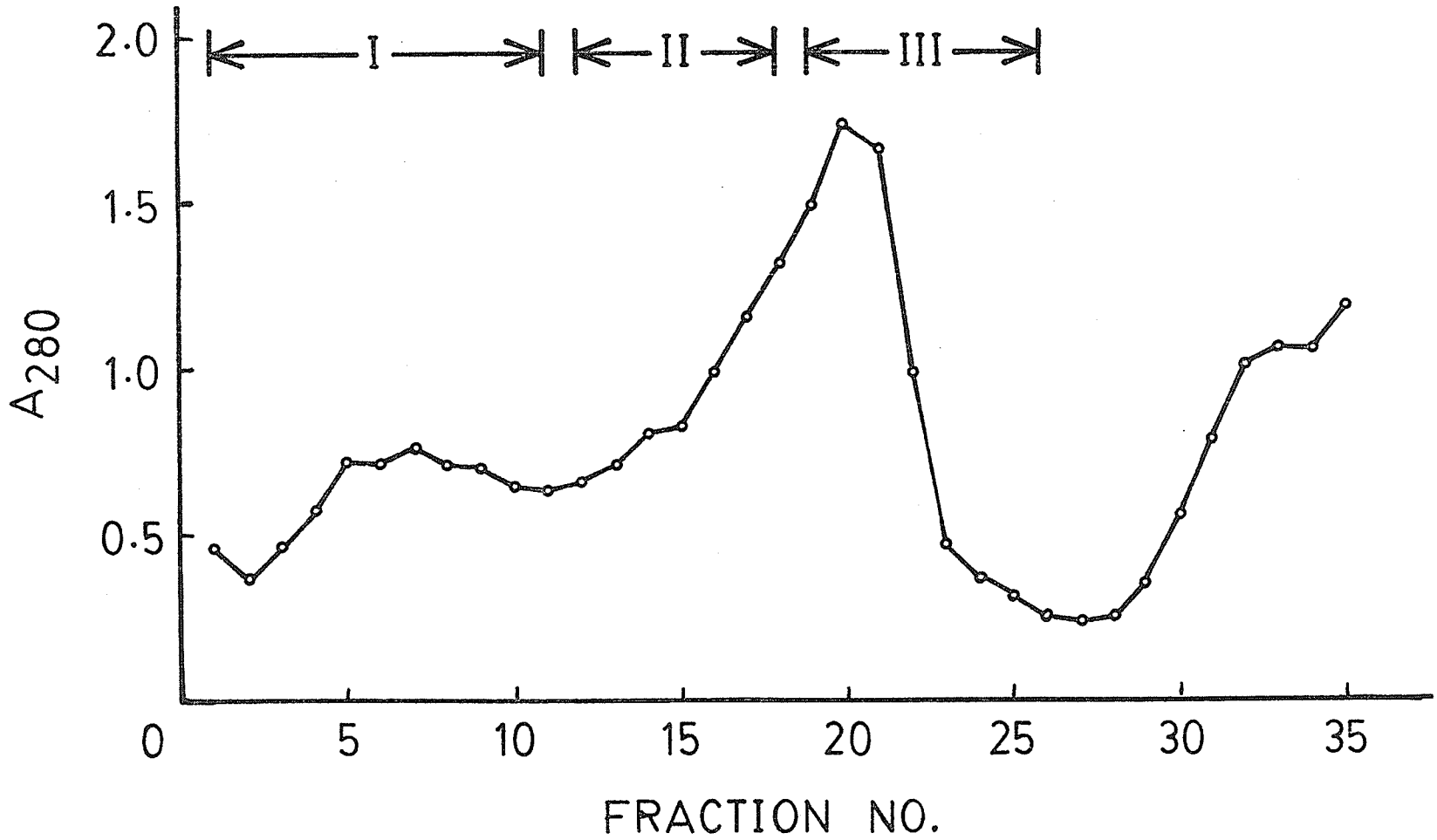

2·0
1·5
1·0
0·5
A_{280}
I
II
III
0
5
10
15
20
25
30
35
FRACTION NO.

Figure 4

Protein profile of total membrane of M. xanthus A^-S^- obtained by sucrose grandient centrifugation formed with 3 ml of 60% sucrose, 6 ml of 50%, 6 ml of 45%, 6 ml of 40%, 6 ml of 35%, 6 ml of 30% and 2 ml of membrane sample. Fractions (39 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm. Fraction I, II and III were obtained by pooling the factions indicated by the bars.

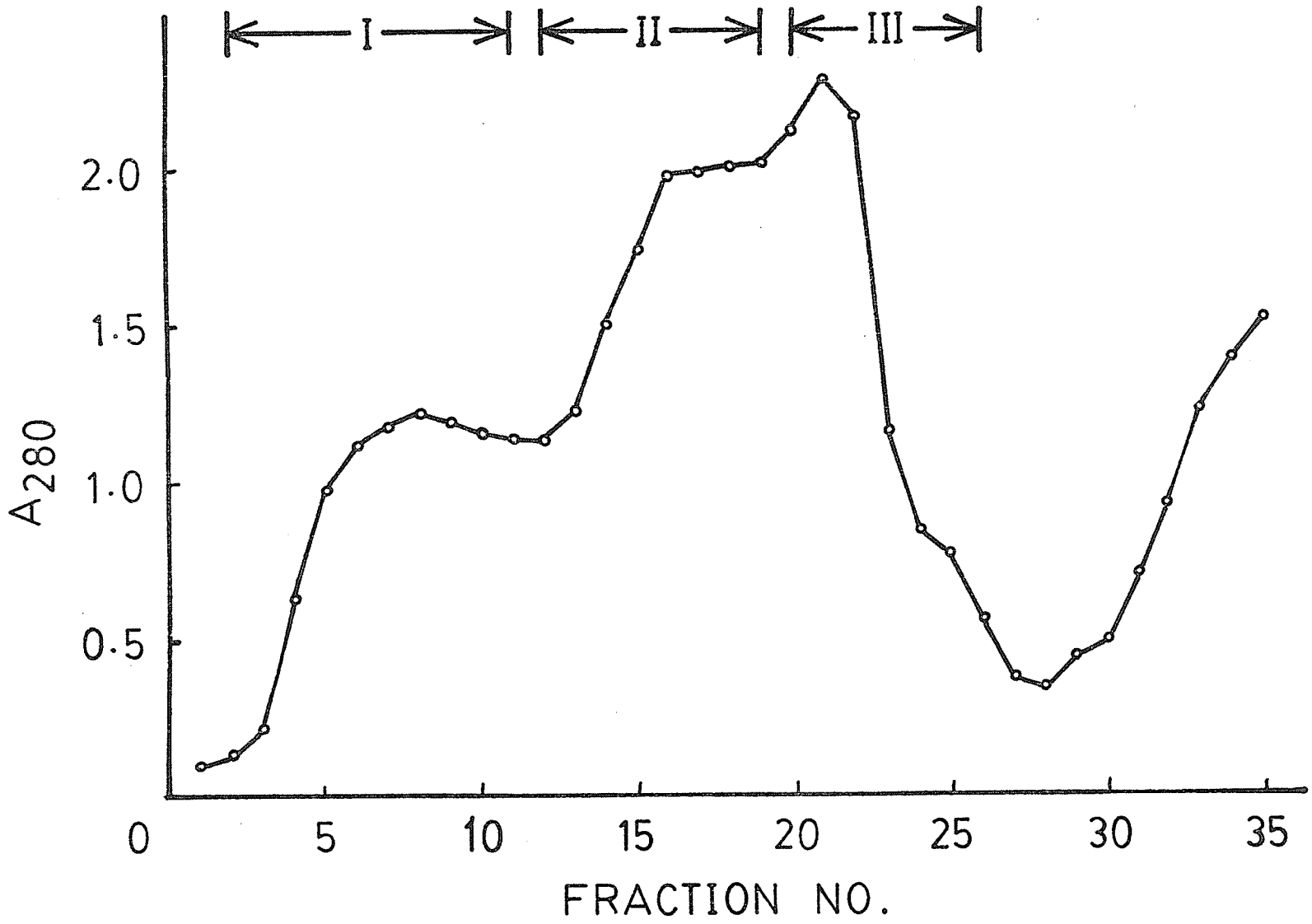
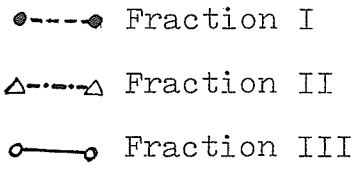
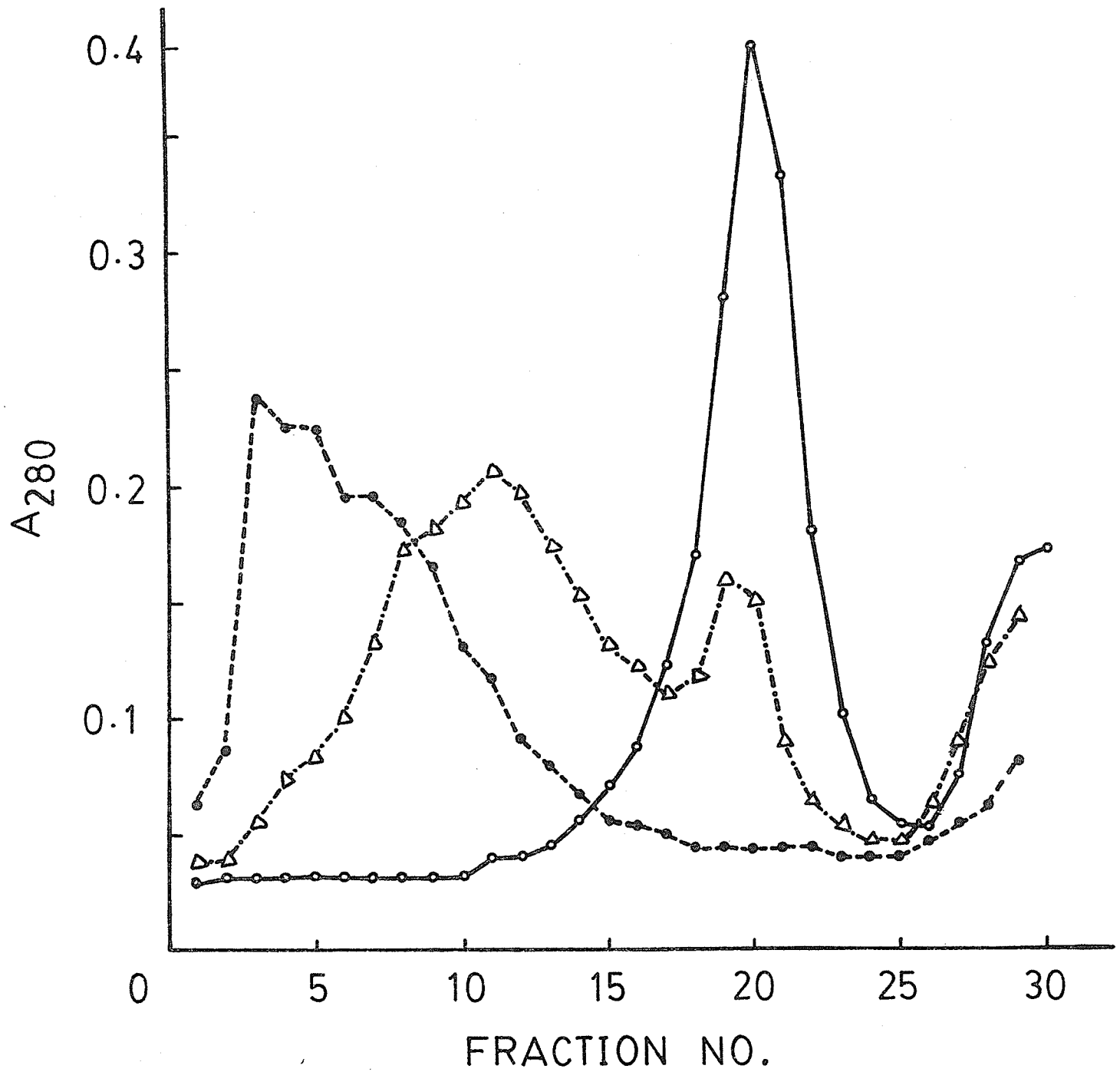

I
II
III
2·0
1·5
1·0
0·5
A_{280}
0
5
10
15
20
25
30
35
FRACTION NO.

Figure 5

Protein profile of pooled membrane fractions I, II and III of M. xanthus A^+S^+ obtained by independent centrifugations in sucrose gradients formed with 1 ml of 60% sucrose, 2 ml of 50%, 2 ml of 45%, 2 ml of 40%, 2 ml of 35%, 2 ml of 30% and 0.5 ml of sample. Centrifugation was done at 25,000 rpm for 18 hours in a Beckman SW41 rotor. Fraction (13 drops) were collected and diluted 2 fold before measuring the absorbance at 280 nm.




0.4
0.3
0.2
0.1
A_{280}
0
5
10
15
20
25
30
FRACTION NO.

Chemical Composition of Isolated Membranes

Some of the properties of the fractions are shown in Table 1. There was 2-3 times more protein in Fraction I than Fraction III. The protein recovered in the fractions was approximately 40%. Additional protein was found at the top of the gradient and may have included membrane protein solubilized during centrifugation. The KDO content, indicative of outer membranes, was essentialy the same in all three fractions (Table 1).

To determine the specificity of the thiobarbituric acid method for KDO (Methods) various samples were subjected to the test. The absorption spectrum of the pinkish coloured product formed by the assay procedure was taken between 430 and 590 nm (Methods). The results are shown in Figure 6. When purified KDO was assayed the coloured product showed a major absorption peak at 548 nm. E. coli LPS (Sigma) showed a small shoulder at 548 nm and a major peak at 520 nm. M. xanthus LPS, prepared as described in Methods, and Calf thymus DNA (Sigma) when subjected to the same treatment yielded a major absorption peak at 520 nm. The result show that the thiobarbiturate assay method is not specific for KDO and that the peak absorbance may shift depending upon the source of the sample. The anomalous results presented in Table 1 depicting the distribution of KDO in all fractions may be due to shifting of the absorbance peak or to contamination of fractions with DNA.

Consequently, a more specific method employing Carbocyanine was used. (Methods) By this method it was shown that

Table 1

Chemical compositions of isolated membrane fractions of M. xanthus A^+S^+, A^+S^-, A^-S^+, A^-S^- obtained by French Pressure Cell method (Method I).

Fraction	Buoyant Density (g/ml)	% Protein	KDO[a] (u mole KDO/ mg protein)	LPS[b] (mg)
A^+S^+				
I	1.209	43.1	0.92	6.1
II	1.170	39.8	1.29	12.1
III	1.148	17.1	1.45	43.5
Total membrane		(100)		152.6
A^+S^-				
I	1.190	36.2	3.50	
II	1.163	41.2	5.50	ND[c]
III	1.145	22.6	4.00	
Total membrane		(100)		
A^-S^+				
I	1.191	51.5	0.76	
II	1.185	25.3	0.93	ND[c]
III	1.156	23.2	0.70	
Total membrane		(100)		
A^-S^-				
I	1.191	45.0	0.39	
II	1.163	31.6	0.49	ND[c]
III	1.158	23.4	0.42	
Total membrane		(100)		

[a] 2-keto-3-deoxyoctanoic acid

[b] Lipopolysaccharide determined by Carbocyanide method

[c] Not determined

Figure 6

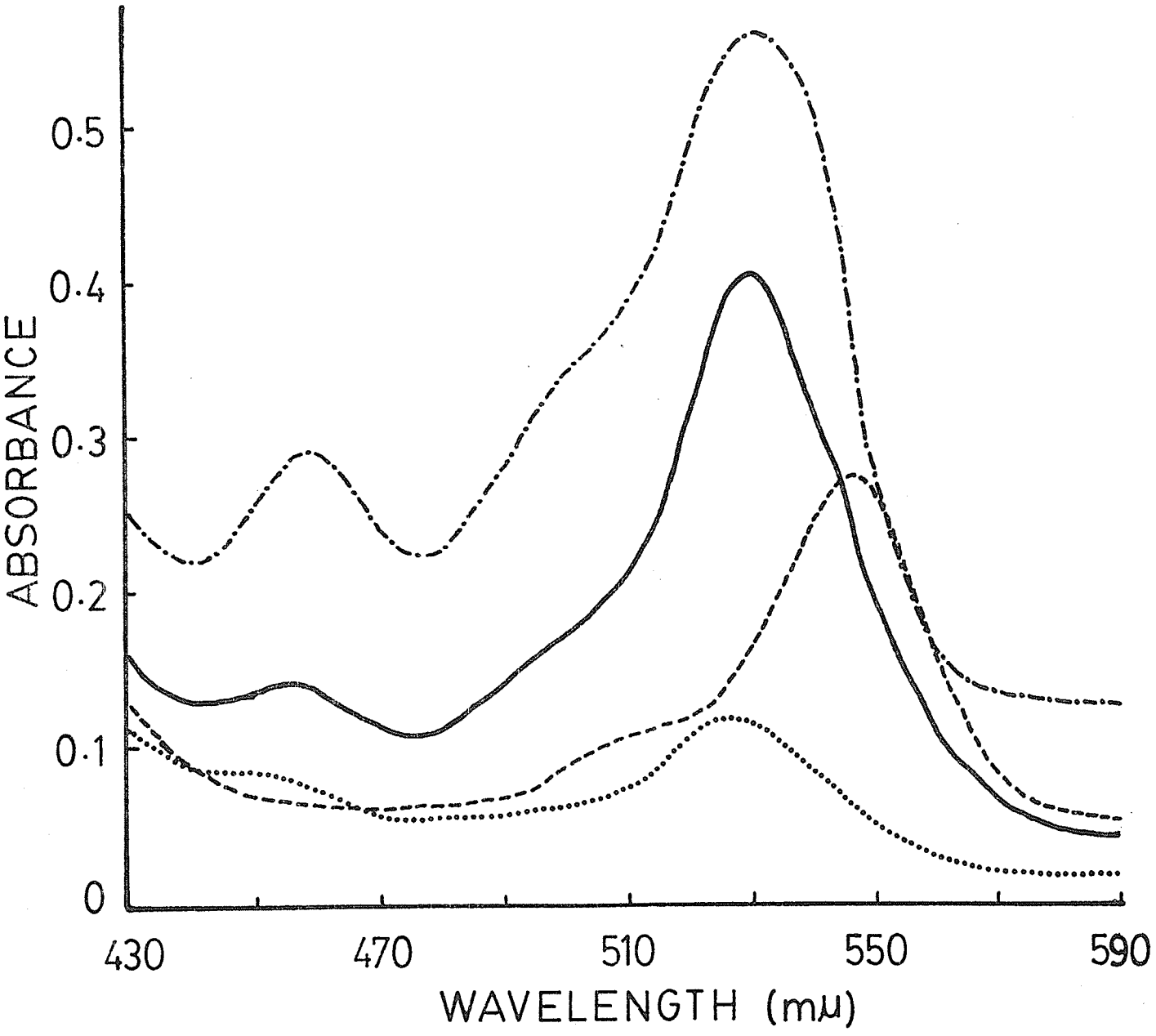
Absorption spectra of coloured product of M. xanthus LPS, KDO, E. coli LPS and DNA formed by thiobarbituric acid assay procedure.

-·-·-·- DNA

——— E. coli LPS

- - - - - KDO

····· M. xanthus LPS


ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0
430
470
510
550
590
WAVELENGTH (mμ)

Fraction III of A^+S^+ contained 7 times more LPS than Fraction I (Table 1) and that 59% of total LPS was lost during isolation. This was likely due to the high concentration of EDTA used during cell breakage. Rogers et al (1969) showed that treatment of isolated cell envelopes of Pseudomonas aerogenosa with EDTA caused the release of a protein-LPS complex. A similar method, omitting EDTA, was unsuccessful since the total membrane could not be separated into different fractions.

The fact that Fraction III was enriched in LPS indicates the fraction consists of outer membrane, whereas Fraction I may consist of cytoplasmic membrane while Fraction II may represent an intermediate fraction of unseparated outer and cytoplasmic membranes.

Enzymes Activities Associated with the Isolated Membrane Fraction Obtained by Method I

To further characterize and test the purity of the three fractions the distribution of succinate dehydrogenase and NADH oxidase, enzymes characteristic of the cytoplasmic membrane, were assayed. The results in Table 2 show that NADH oxidase and succinate dehydrogenase activities were 5 to 10 times higher in Fraction I than in Fraction III in all 4 strains of M. xanthus. These observations and those of chemical compositions (Table 1) led us to conclude that Fraction I represented the cytoplasmic membrane and Fraction III the outer membrane. Fraction II was likely the unresolved cytoplasmic and outer membrane complex.

Table 2

Enzyme activities of isolated membrane fractions of M. xanthus A^+S^+, A^+S^-, A^-S^+, A^-S^- obtained by Method I.

Fraction	NADH* Oxidase	Succinate* Dehydrogenase
A^+S^+		
I	.087	2.51
II	.088	3.34
III	.009	0.31
Total membrane	.027	0.48
A^+S^-		
I	.068	1.67
II	.016	1.27
III	.012	0.96
Total membrane	.050	0.80
A^-S^+		
I	.113	2.23
II	.031	1.39
III	.014	0.34
Total membrane	.041	0.81
A^-S^-		
I	.085	1.96
II	.017	0.95
III	-0-	0.29
Total membrane	.113	0.67

*Specific activities are expressed as micromoles/mg protein/minute.

SDS-polyacrylamide Gel Electrophoresis of Membrane Fractions Obtained by Method I

Polypeptides of the membrane fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Figure 7). The cytoplasmic membrane (Fraction I) contained approximately 28 polypeptide bands; the outer membrane (Fraction III) approximately 24 bands. Fraction II appeared to have a protein pattern of the combination of Fraction I and III. Seven major bands (as indicated by the arrows) appeared more prominantly in outer membranes than in cytoplasmic membranes as judged by the intensity of staining (open arrows, Fig. 7). In contrast seven protein bands stained more intensely in cytoplasmic membranes than in the outer membranes (solid arrows, Fig. 7). No marked difference was observed between the gel electrophoresis pattern of outer and cytoplasmic membranes. They appeared to differ in the staining intensity of the individual bands.

The gel electrophoresis polypeptide pattern of outer membrane fraction of strains A^+S^+, A^+S^-, A^-S^+ and A^-S^- are shown in Figure 8. To our suprise the protein patterns of the three mutants did not show any significant difference from the wild type A^+S^+. The differences observed were due to different protein loads applied to the gels.

Cells at different stages during fruiting development were harvested and treated according to Method I. Figure 9 shows the protein profiles after sucrose gradient centrifugation of total membranes isolated from cells that had undertaken fruiting body formation for 0, 12, 24 and 36 hours.

Figure 7

SDS-polyacrylamide gel electrophoresis of membrane fractions from M. xanthus A^+S^+. Solubilized membrane fraction were subjected to electrophoresis on a 12.9% acrylamide and stained with commassic brilliant blue as described in Methods.

I : Fraction I (cytoplasmic membrane)

II : Fraction II (unresolved membrane complex)

III : Fraction III (outer membrane)

◆ : bands that are more intensely stained in outer membrane than cytoplasmic membrane.

◇ : bands that are more intensely stained in cytoplasmic membrane than outer membrane.

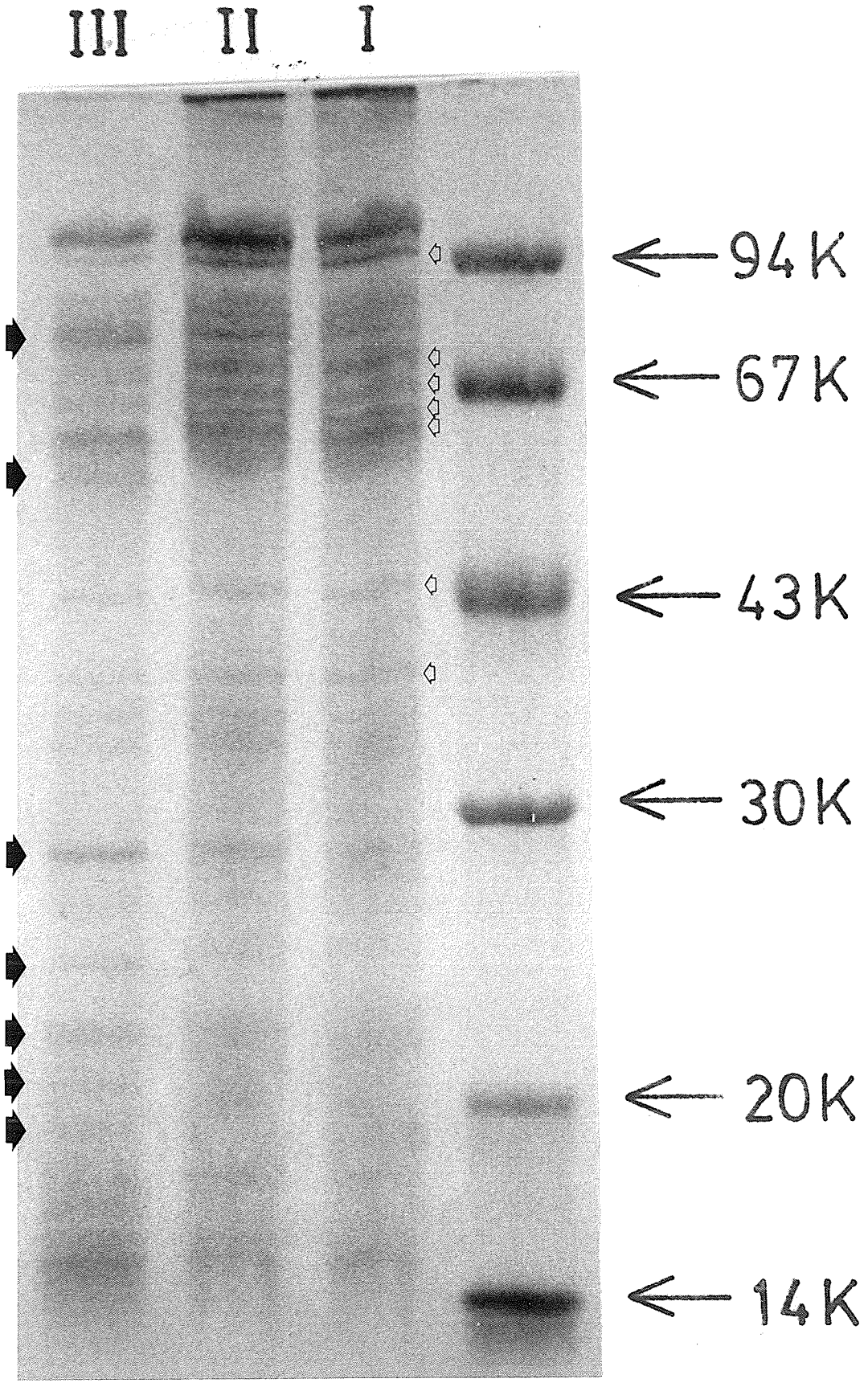

III
II
I
94K
67K
43K
30K
20K
14K

Figure 8

SDS-polyacrylamide gel electrophoresis of outer membrane fractions from M. xanthus A^+S^+, A^+S^-, A^-S^+, A^-S^-.

++ : M. xanthus A^+S^+

+- : M. xanthus A^+S^-

-+ : M. xanthus A^-S^+

-- : M. xanthus A^-S^-

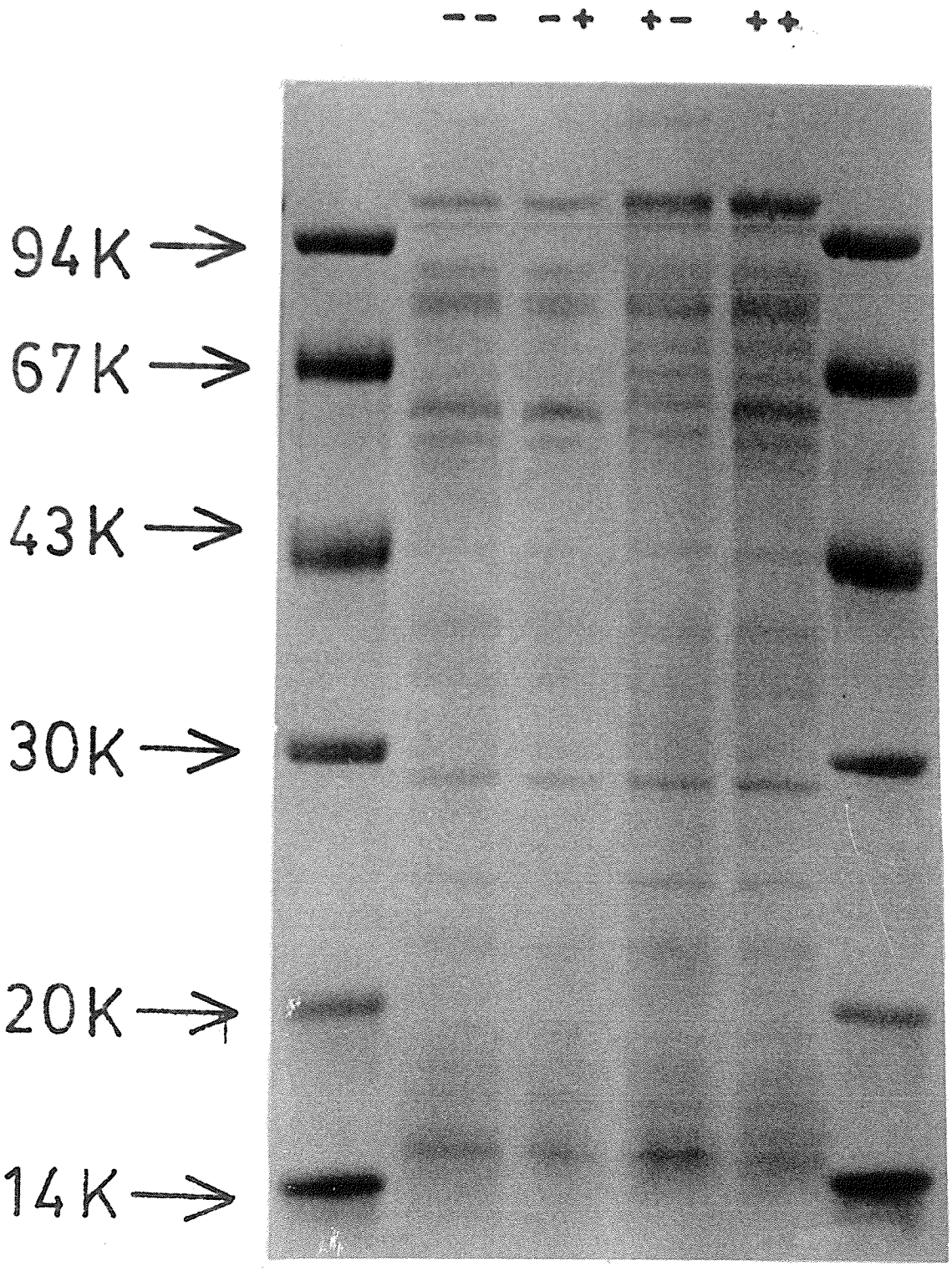

-- -+ +- ++
94K
67K
43K
30K
20K
14K

Figure 9

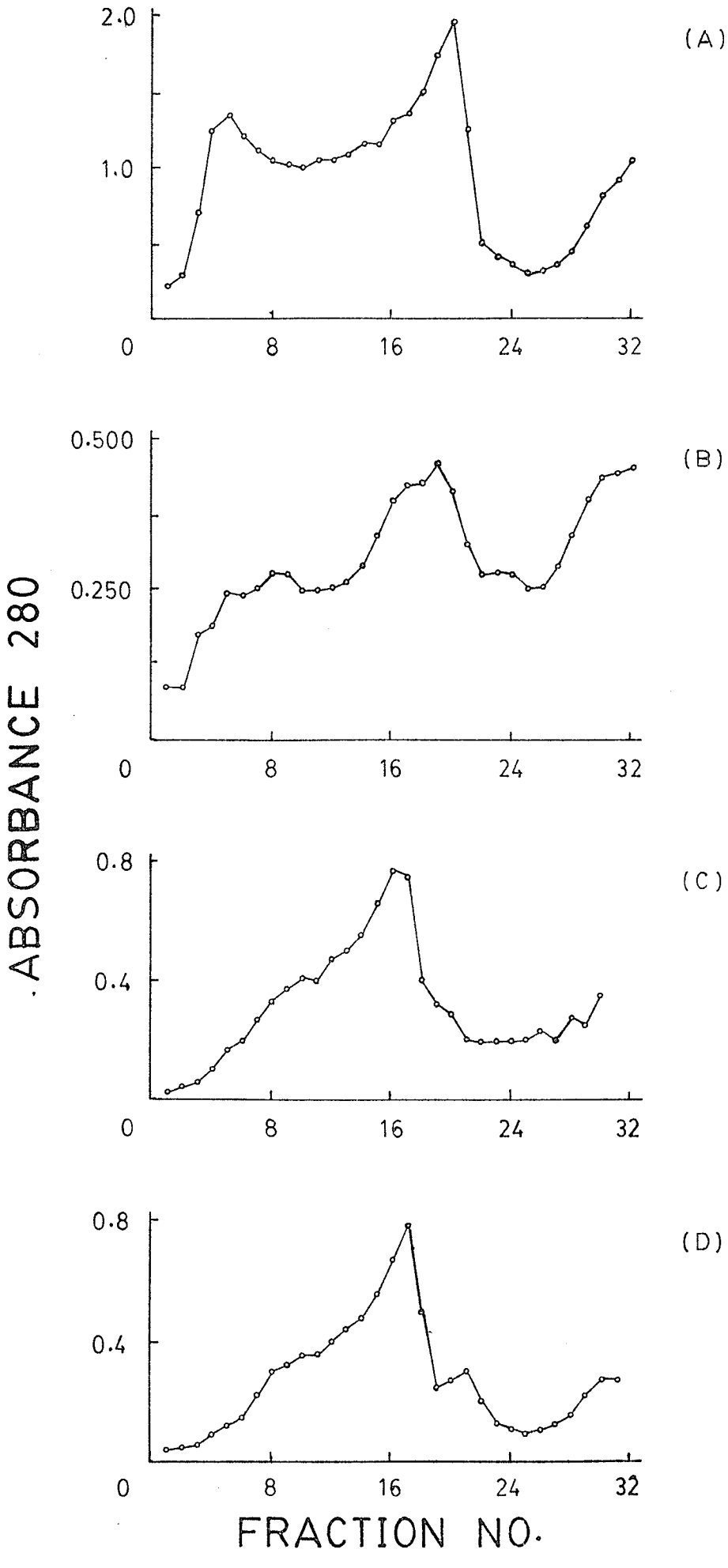
Protein profiles after sucrose gradient centrifugation of total membranes of M. xanthus A^+S^+ obtained by Method I during various stages of Fruiting body development.

(A) 0 hours

(B) 12 hours

(C) 24 hours

(D) 36 hours


(A)
2.0
1.0
0
8
16
24
32
(B)
0.500
0.250
0
8
16
24
32
(C)
0.8
0.4
0
8
16
24
32
(D)
0.8
0.4
0
8
16
24
32
ABSORBANCE 280
FRACTION NO.

After 48 hours of development cells were difficult to extract and the membranes extracted from these cells did not readily separate into different fractions. The heavier peak (Fraction 6 in Fig. 9A) became less distinct when membranes were extracted from cells that had undertaken development for longer periods. This peak appears as a shoulder in membranes isolated from cells that had been allowed to develop for 36 hours. The lighter peak also shifts to the left indicating that it has become denser.

Figure 10 shows the SDS gel electrophoresis protein pattern of outer membranes (Fraction III) from cells at different stages of development. The amounts of several proteins, as indicated by the staining intensity of the bands, changes during the development process. Bands that become more prominent with development are bands A, E and F. (Fig. 10) Whereas bands C and D become less prominent. One band, B, increased in amount in the early stages, but decreased towards the end of development.

Method II (Lysozyme/EDTA/Brij 35 Method)

Membranes were extracted from vegetative cells employing lysozyme, EDTA and Brij 35 with (Method IIa) and without (Method IIA) sonication as described in Methods.

Tsukagoshi and Fox (1971) suggested that sonication could lead to hybridization of outer and inner membranes to form a membrane complex of intermediate density. When sonication was employed, three distinct yellowish brown bands were visible after sucrose gradient centrifugation. The protein profile

Figure 10

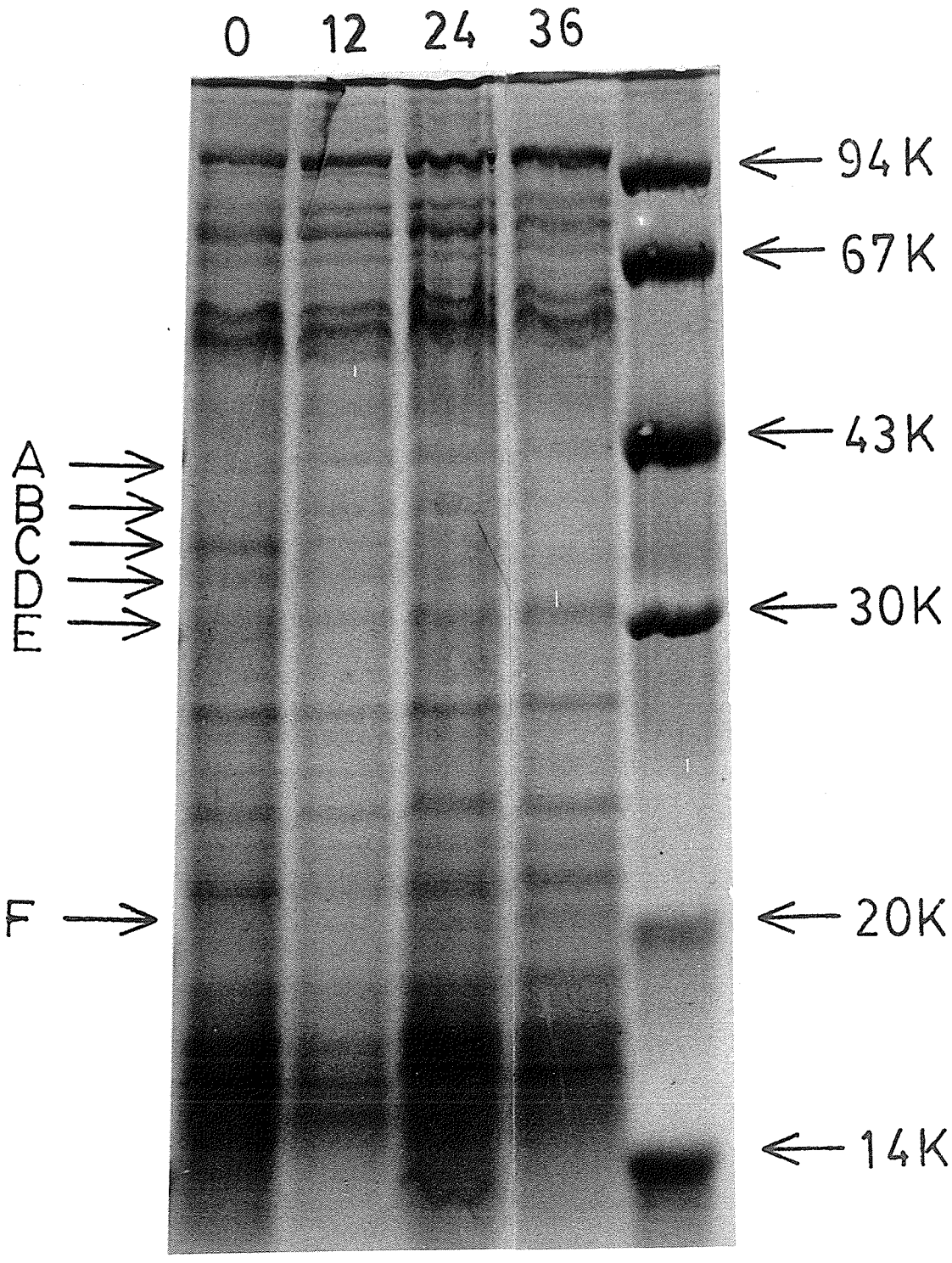
SDS-polyacrylamide gel electrophoresis of outer membranes of M. xanthus A^+S^+ obtained by Method I during various stages of Fruiting body development.

0 : 0 hours

12 : 12 hours

24 : 24 hours

36 : 36 hours


0
12
24
36
94K
67K
43K
30K
20K
14K
A
B
C
D
E
F

is shown in Figure 11. When the sonication step was omitted, five bands appeared after sucrose gradient centrifugation, (Fig. 12). The two heaviest bands had a fainter yellow colour when compared with the three lighter bands. They were pooled as one fraction (Fraction H_1) due to the close proximity of the two bands. The three bands obtained with sonication had identical buoyant densities to the three lightest bands of membranes prepared without sonication (Table 3). The sonication step also led to a decrease in amount of protein recovery in Fraction M and increase in amount of protein recovery in Fraction L as judged by profile in Fig. 11 and 12. When subjected individually to sucrose gradient centrifugation, each pooled fraction resolved into two or three different bands. This indicated the lack of homogenecity within each pooled fraction. This is in marked contrast to membranes from cells extracted by the French press (Method I).

Chemical Compositions of Isolated Membranes Fraction Obtained by Method II

The chemical compositions of the isolated membrane fractions are shown in Table 3. The three fractions obtained by Method II A, i.e., H, M, and L (Fig. 11), and Method II B, i.e., H_2, M, L (Fig. 12) have similar buoyant densities. Since we used 70% (w/v) sucrose in the sucrose gradient in Method II B, the H_1 fraction, which contained granular material, might be the material that pelleted at the bottom during sucrose gradient centrifugation in Method II A.

Figure 11

Protein profile of total membrane of M. xanthus A^+S^+ obtained by discontineous sucrose gradient. Centrifugation (3 ml of 60% sucrose, 6 ml of 50%, 6 ml of 45%, 6 ml of 40%, 6 ml of 35%, 6 ml of 30% and 2 ml of sample). Fractions (35 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm.

Total membrane was obtained by treating the cells with lysozyme/EDTA/Brij 35 and ultrasound sonication (Method IIA). Membrane Fraction H, M and L are pooled from the fractions indicated by the bars.

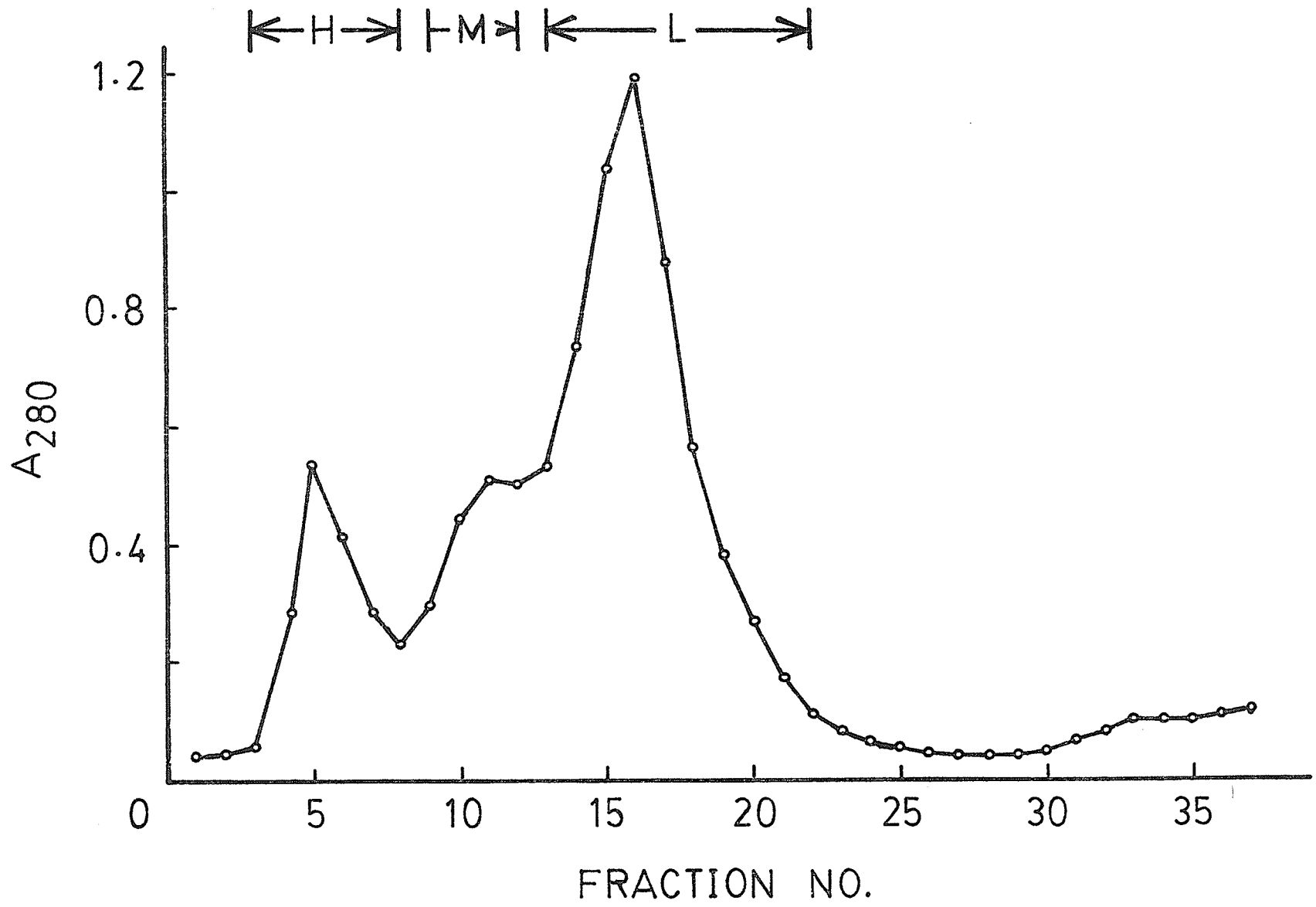

H
M
L
1·2
0·8
0·4
A_{280}
0
5
10
15
20
25
30
35
FRACTION NO.

Figure 12

Protein profile of total membrane of M. xanthus A^+S^+ obtained by discontinuous sucrose gradient centrifugation. (1 ml of 70% sucrose, 5 ml of 60%, 5.5 ml of 55%, 6 ml of 50% 7.5 ml of 45%, 6 ml of 40% and 1.25 ml of sample).

Total membrane was obtained by treating the harvested cells with lysozyme/EDTA/Brij 35 omitting the ultrasound sonication step (Method IIB). Membrane Fraction H_1, H_2, M and L are pooled from the fractions indicated by the bars.

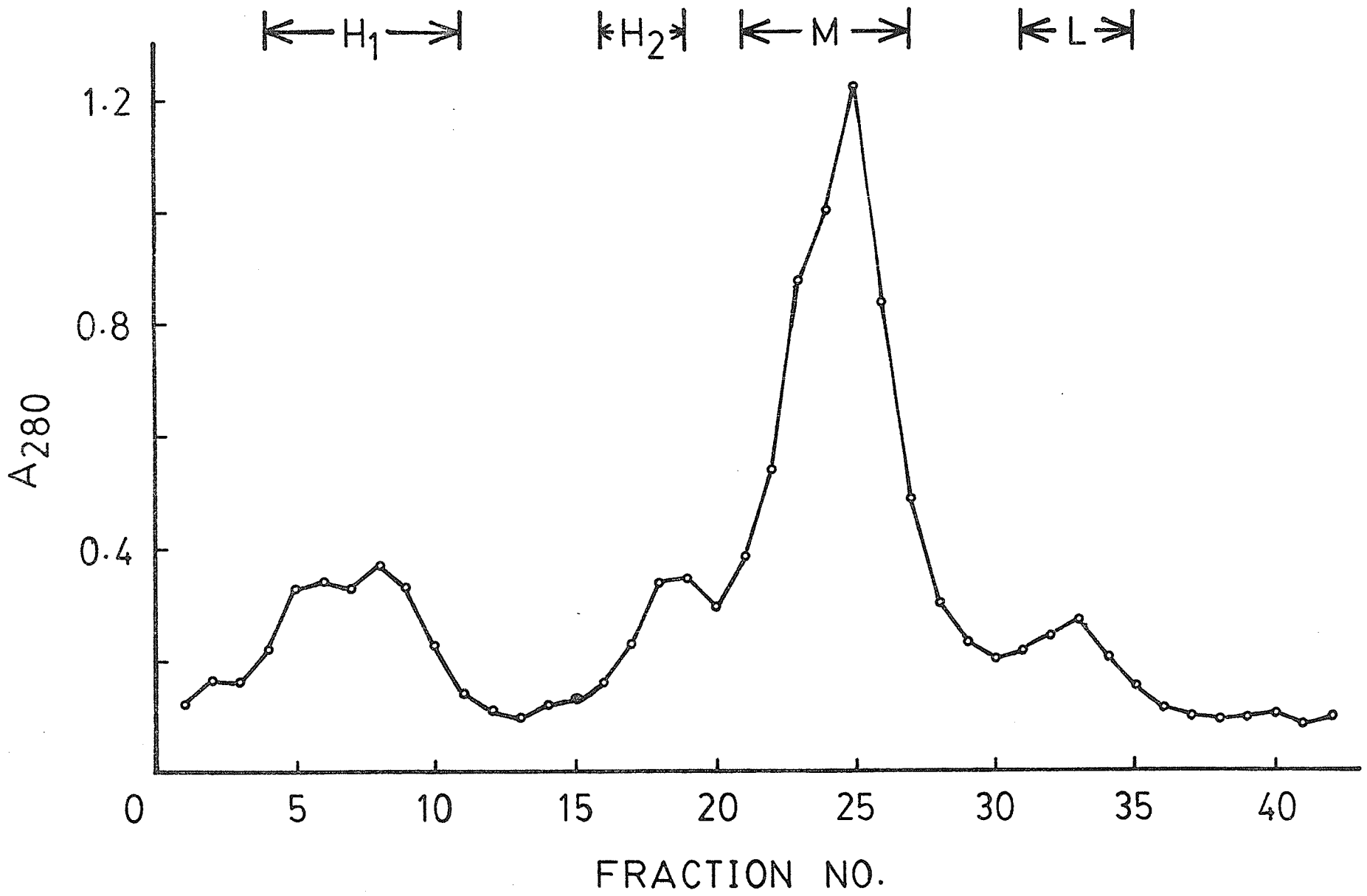

H1
H2
M
L
1.2
0.8
0.4
A280
0
5
10
15
20
25
30
35
40
FRACTION NO.

Table 3

Chemical composition of isolated membrane fractions of M. xanthus A^+S^+ obtained by Method II.

Fraction	Buoyant Density (g/ml)	% Protein	KDO[a] (u mole KDO/ mg protein)	LPS[b] (mg)
(With ultrasound sonication)-Method II A				
H	1.195	26.8	1.16	20.10
M	1.174	24.8	0.64	19.10
L	1.158	48.4	0.98	7.75
Total membrane		(100)		
(Without ultrasound sonication)-Method II B				
H_1	1.231	18.8	1.38	2.50
H_2	1.199	15.9	1.45	2.50
M	1.180	43.0	0.84	14.00
L	1.150	22.3	1.12	6.60
Total membrane		(100)		

[a]Thiobarbituric Acid Assay

[b]Carbocyanine Assay

The distribution of proteins in the H, M and L fractions showed marked difference when isolated by Method II A and II B (Table 3). If Fraction M represents the hybridized membrane fraction, then sonication lead to a decrease of protein recovery in this fraction from 43% to 24.8%.

The specific amount of KDO in Fractions H and L (Method II A) was more or less similar, as were the amount in fractions H_2 and L (Method II B) as shown in Table 3. However there was 2 to 3 times more LPS in L fraction than in H fraction isolated by Method II A or II B, suggesting that the lighter fraction was enriched with outer membrane.

Enzymes Activities of Membrane Fraction

When membrane fractions were isolated by Method II A, the H fraction contained less NADH oxidase and succinate dehydrogenase activities than the L fraction (Table 4). However, when isolated by Method II B the opposite occured, i.e., the H_2 fraction showed slightly higher NADH oxidase and succinate dehydrogenase activities than the L fraction.

Figure 13 shows an electropherogram of the three membrane fractions obtained by Method II A. There was no noticeable difference in banding patterns of Fractions L and M, although the staining intensity of some bands were quite different.

Method III (Reichenbach-Kageyama-Schnaitman Method).

We were unable to obtain spheroplasts by treating the cells with lysozyme and EDTA in sucrose solution according to the method of Mizuno and Kageyama (1978). When the method

Table 4

Enzymes activities of isolated membrane fraction of M. xanthus A^+S^+ isolated by Method II.

Fraction	specific activity (micromoles/mg protein/minute)	
	NADH oxidase	succinate dehydrogenase
Method IIA		
H	.004	3.50
M	.006	9.80
L	.007	19.95
Total membrane	.003	3.85
Method IIB		
H1	0	0.32
H2	.004	4.15
M	.001	9.58
L	.003	3.89
Total membrane	.004	0.49

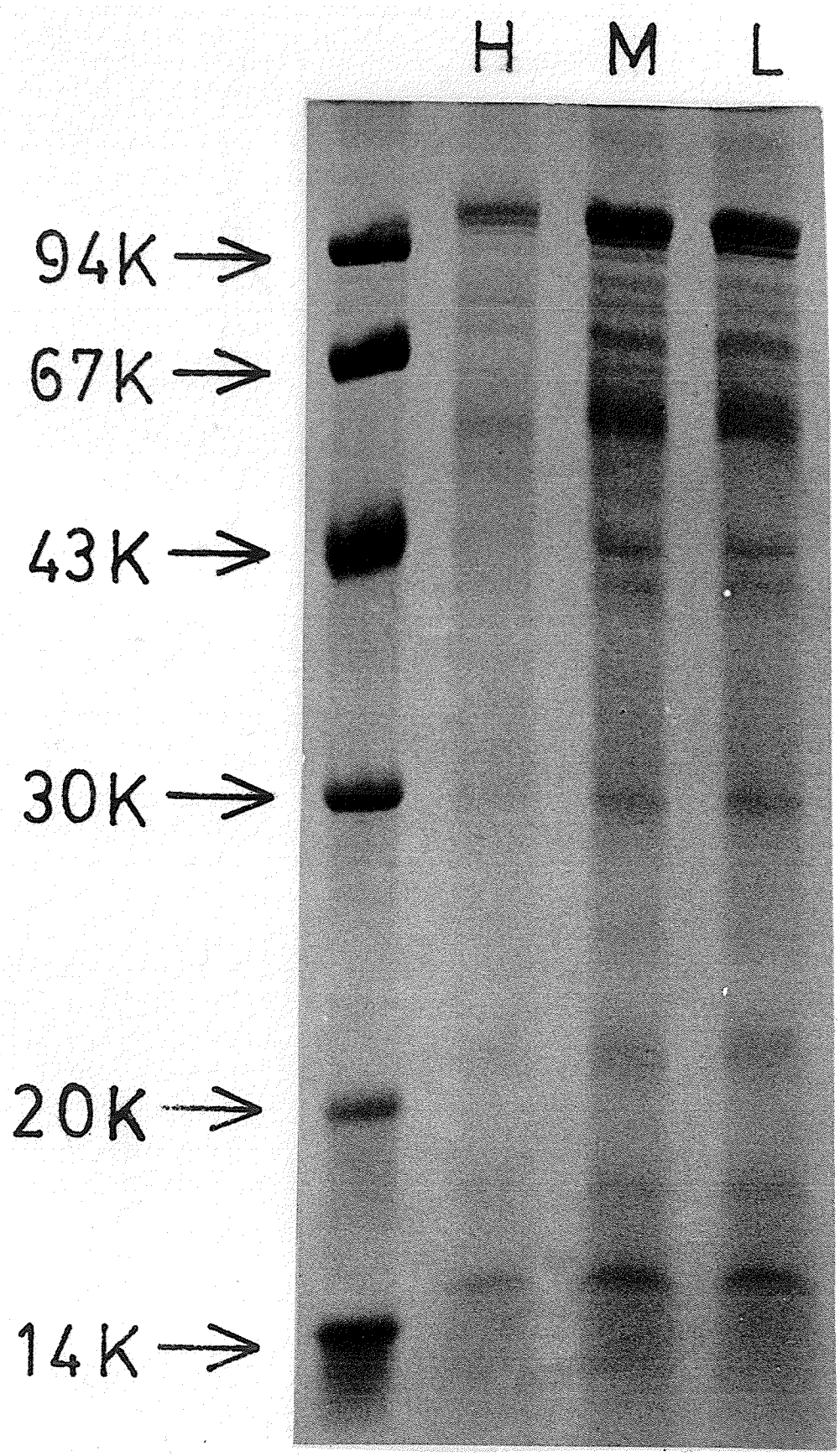
Figure 13

SDS-polyacrylamide gel electrophoresis of membrane fractions of *M. xanthus* A^+S^+ obtained by Method IIA (with sonication).

H : Fraction H (cytoplasmic membrane)

M : Fraction M (intermediate complex)

L : Fraction L (outer membrane)


H
M
L
94K
67K
43K
30K
20K
14K

was modified as described by Irschik and Reichenbach by replacing sucrose with glycerol, 90% of the cells were converted to spheroplast. Low speed centrifugation separated spheroplasts from the supernatant. The spheroplasts could not be lysed by osmotic shock or by treatment with Brij 35, and had to be lysed in a French pressure cell. This fraction was designated Rsph (Reichenbach spheroplast fraction). The supernatant of the low speed centrifugation should be enriched in outer membrane fragments (Kageyama, 1978). Consequently, material in the supernatant, Rsup (Reichenbach supernatant) was pelleted by high speed centrifugation (150,000 x g, 90 min) and subjected to sucrose gradient centrifugation. The sedimentation profile is shown in Figure 14. Three bands were visible, but only the heaviest band gave significant absorbance at 280 nm. A fraction designated RH (Reichenbach Heavy fraction) was pooled as shown in Figure 14. When the spheroplast lysate (Rsph) was centrifuged in a sucrose gradient, three bands were observed, the 2 lighter bands absorbing strongly at 280 nm. The lightest band had a intense brown colour. The sedimentation profile and the fractions that were pooled to give yield RM (Reichenbach Middle) and RL (Reichenbach Light) are shown in Figure 15. From Figure 14 we can see that Rsup fraction is quite homogenous yielding only 1 major peak which was pooled into the RH fraction. A small amount of material whose densities corresponded to RM and RL fractions in the spheroplast lysate was observed.

Figure 14

Protein profile of Rsup Crude fraction after sucrose gradient centrifugation (4 ml of 60% sucrose, 9.5 ml of 55%, 9.5 ml of 50%, 3.5 ml of 45%, 2.5 ml of 40% and 2.5 ml sample). Ksup Crude fraction was obtained by Method III. Fractions (35 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm.

Membrane fraction A was pooled from the fractions indicated by the bars.

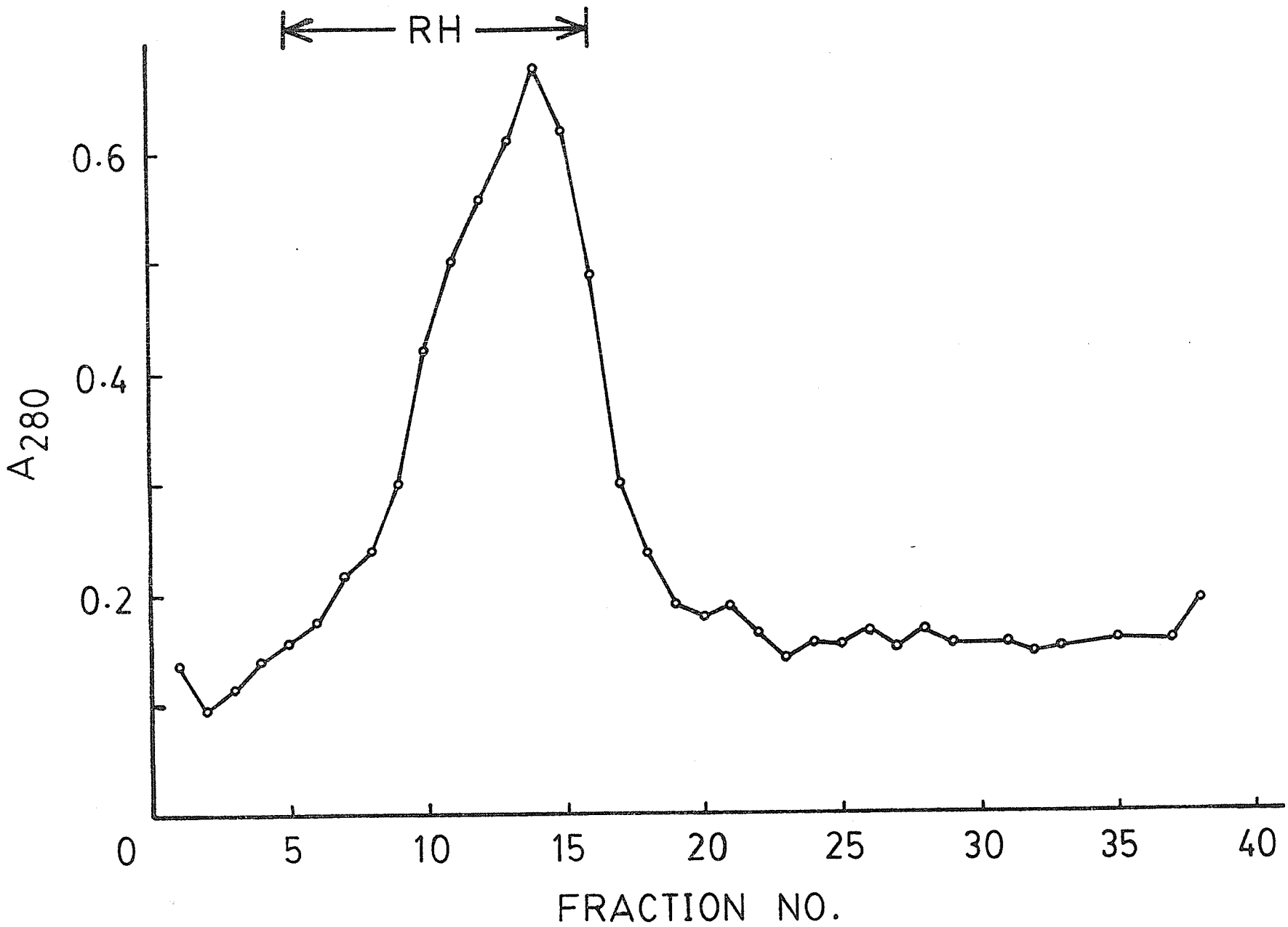
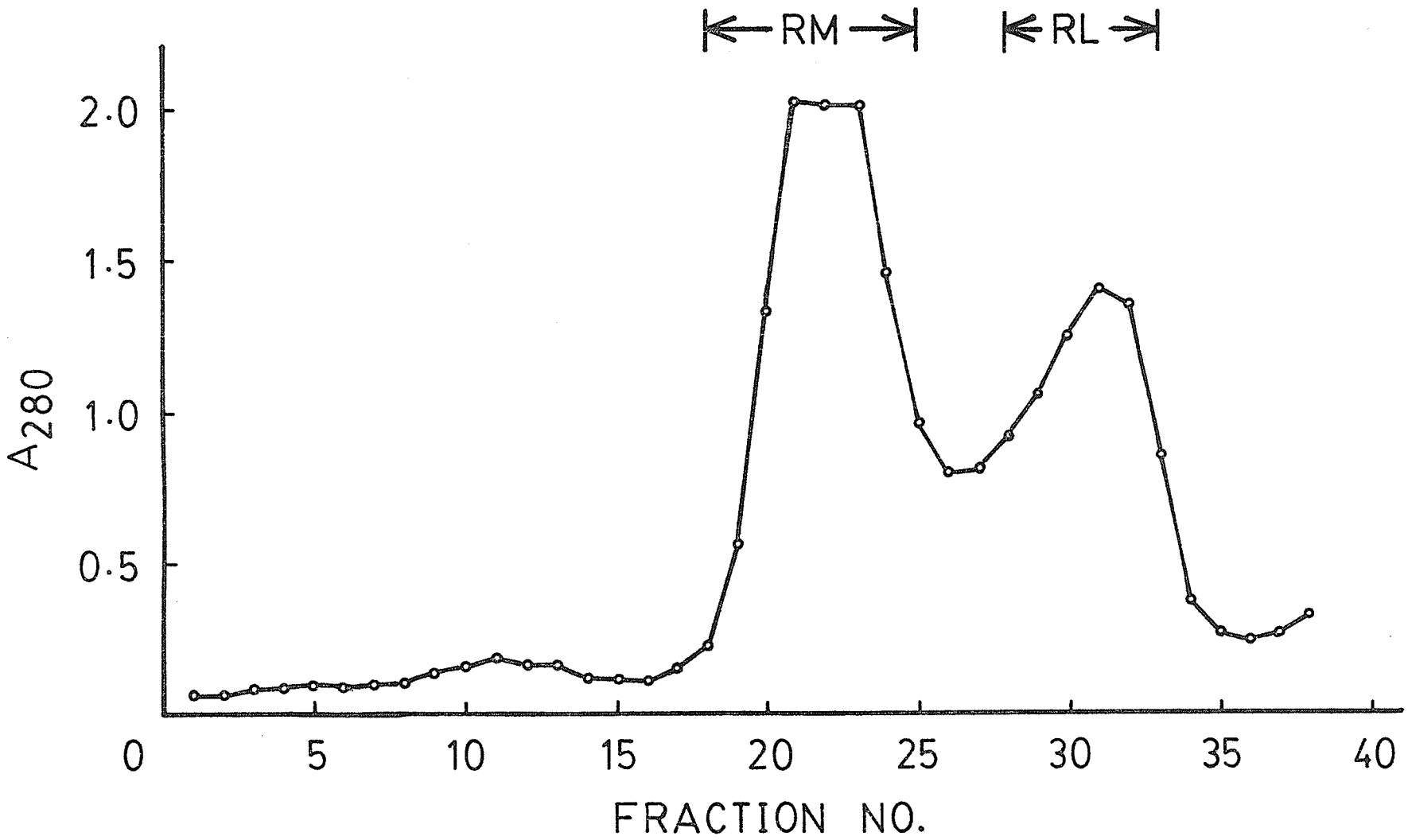

RH
0.6
0.4
0.2
A_{280}
0
5
10
15
20
25
30
35
40
FRACTION NO.

Figure 15

Protein profile of Rsph fraction after sucrose gradient centrifugation (4 ml of 60% sucrose, 9.5 ml of 55%, 9.5 ml of 50%, 3.5 ml of 45%, 2.5 ml of 40% and 2.5 ml sample). Rsph was obtained by Method III. Fractions (35 drops) were collected and diluted 10 fold before measuring the absorbance at 280 nm.

Membrane fractions RM and RL were pooled from the fractions indicated by the bars.


RM
RL
2·0
1·5
1·0
0·5
A_{280}
0
5
10
15
20
25
30
35
40
FRACTION NO.

Chemical Composition of the Isolated Membrane Fraction by Method III

As shown in Table 5 the Rsup Crude extract contains more LPS than Rsph Crude and RH fraction has twice the amount of LPS than the RL fraction. This indicates that RH is the outer membrane; RL, the cytoplasmic membrane and RM, the hybrid intermediate. Table 6 shows the distribution of the enzymatic activities in different preparations. The purified outer membrane fraction (RH) contained about 5 times less NADH oxidase activity than RL and succinate dehydrogenase activity was found exclusively in the Rsph Crude fraction. The results therefore agree with that of Kageyame (1978).

An electropherogram of fraction RH, RM and RL is shown in Figure 16. There are 6 bands missing from the outer membrane fractions (RH) that are present in the cytoplasmic membrane fraction (RL) (solid arrows, Fig. 16). Also the outer membrane fraction contains two bands that are missing from cytoplasmic membrane fraction (open arrows, Fig. 16).

Method IV (Triton X-100 extraction)

This method was based on the solubility properties of the different membrane in detergent. Under the experimental condition cytoplasmic membrane becomes completely dissociated in the presence of the nonionic detergent, Triton X-100. Cell lysate subjected to Triton X-100 treatment yielded a Triton X-100 soluble fraction (TSF) and a Triton X-100 insoluble fraction (TIF). According to Schnaitman (1971 a & b), the TSF fraction contained outer membrane. The chemical

Table 5

Chemical compositions of pooled membrane fractions and crude fractions of M. xanthus obtained by Method III.

Fraction	Buoyant Density (g/ml)	% Protein	KDO[a] (u mole KDO/ mg protein)	LPS[b] (mg)
RH	1.192	32.6	.018	2.50
RM	1.176	39.4	.044	1.33
RL	1.154	28.0	.017	1.28
Rsph			.014	3.75
Rsup			.009	6.42

[a]Thiobarbituric Acid Assay (Methods)

[b]Carbocyanine Assay (Methods)

Table 6

Enzyme activities of pooled membrane fraction and crude fractions of M. xanthus obtained by Method III.

Fraction	Specific activity (micromoles/mg protein/minute)	
	NADH oxidase	succinate dehydrogenase
RH	.010	0
RM	.137	0
RL	.054	0
Rsph	.015	1.130
Rsup	.004	.007

Figure 16

SDS-polyacrylamide gel electrophoresis of membrane fraction RL, RM and RH obtained by Method III.

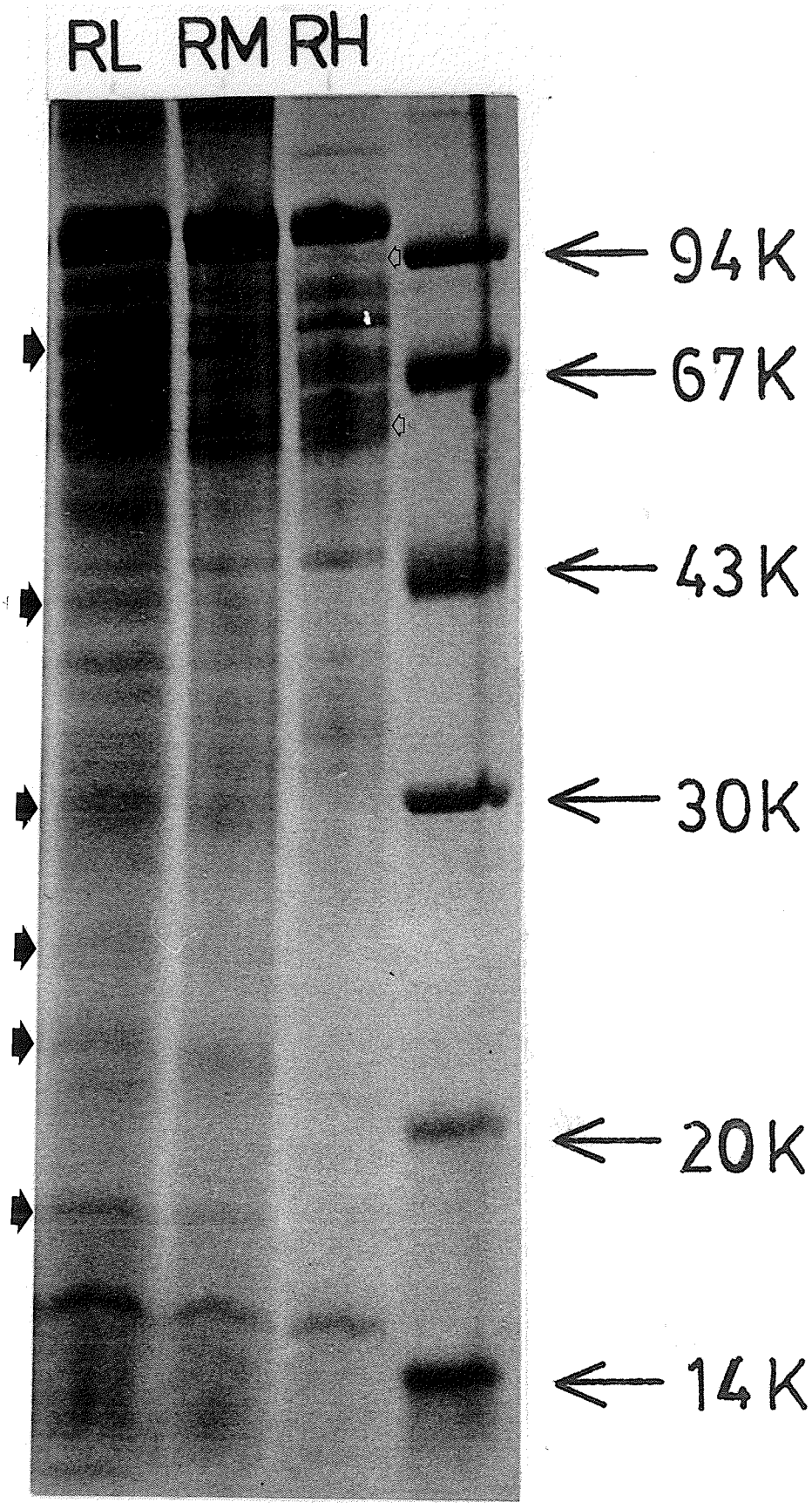
RL : Fraction RL

RM : Fraction RM

RH : Fraction RH

⬟ : bands that are found in Fraction RL but not in RH

◇ : bands that are found in Fraction RH but not in RL


RL
RM
RH
94K
67K
43K
30K
20K
14K

composition of TIF and TSF are shown in Table 7. The protein content in each fraction was almost the same. TIF has 2 to 3 times more LPS content than TSF. From Table 8, it can be seen that TSF fraction contained all the succinate dehydrogenase activity. No detectable NADH oxidase activity could be found in either TIF or TSF fractions. The results agree with the suggestion of Schnaitman (1971 a & b) that TIF represents outer membrane while TSF represents cytoplasmic membrane.

The electropherograms (Fig. 17) of TIF and TSF fractions show marked differences. As shown in Figure 17 the outer membrane fraction (TIF) has 6 bands that absent from TSF (solid arrows, Fig. 17) while the TSF (cytoplasmic membrane) has 4 bands that are missing from TIF fraction (open arrows, Fig. 17).

When comparing the electrophoretic profiles of fractions obtained by Method III and IV we were unable to notice any correlation in missing bands.

Table 7

Chemical compositions of Triton X-100 soluble and Triton X-100 insoluble fractions of M. xanthus A^+S^+.

Fraction	Buoyant Density (g/ml)	% Protein	KDO (u mole KDO/ mg protein)	LPS (mg)
TIF	-	44	.003	17.40
TSF	-	56	.001	6.36

Table 8

Enzyme activities of Triton X-100 soluble and Triton X-100 unsoluble fractions of M. xanthus A^+S^+.

Fraction	Specific activity (micromoles/mg protein/minute)	
	NADH oxidase	succinate dehydrogenase
TIF	0	0
TSF	0	1.120
Crude	.010	.446

Figure 17

SDS-polyacrylamide gel electrophoresis of Triton X-100 soluble and Triton X-100 insoluble fraction of M. xanthus A^+S^+.

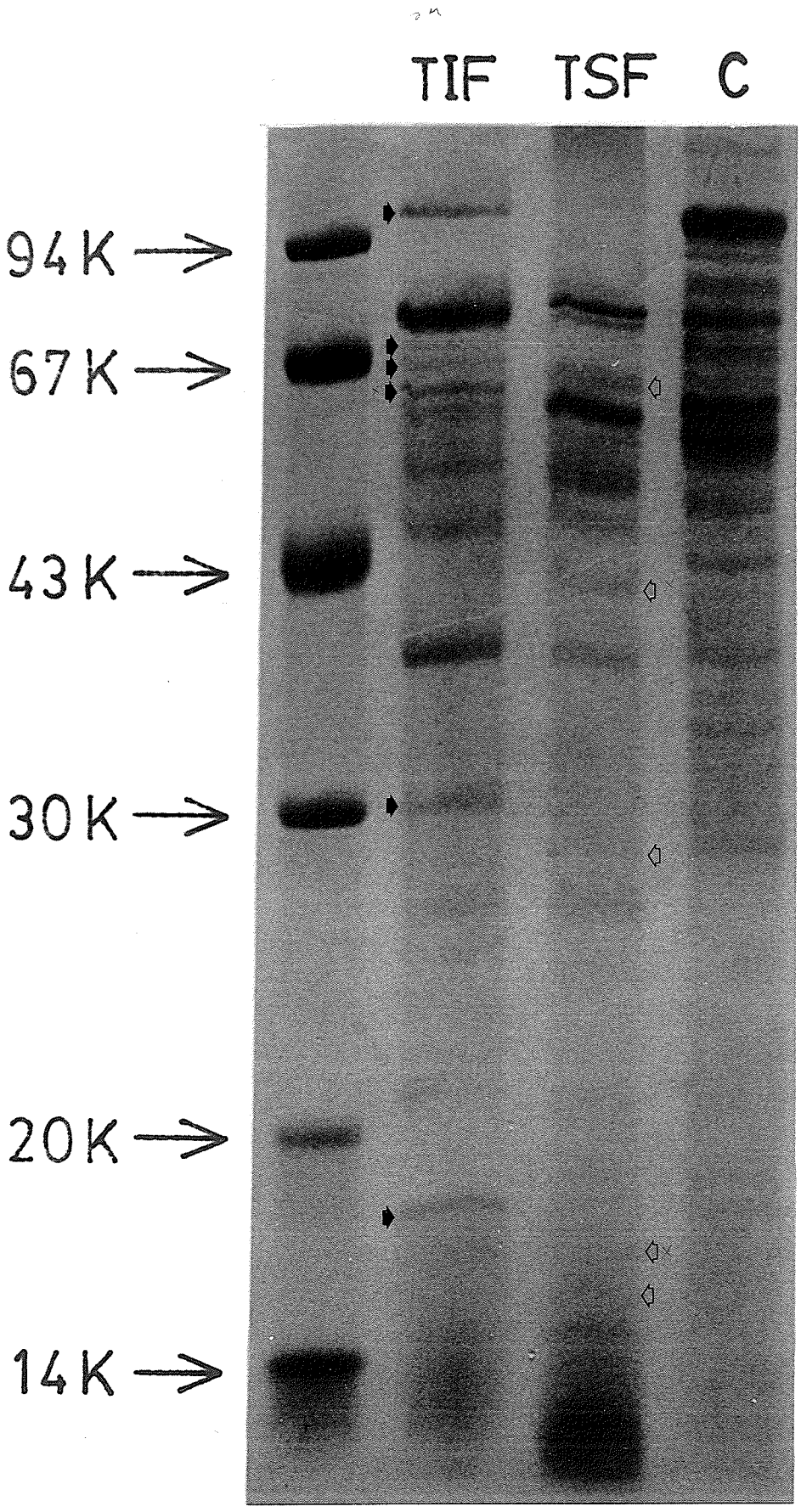
TIF : Triton X-100 insoluble fraction

TSF : Triton X-100 soluble fraction

C : Crude membrane fraction

◗ : bands that are found in TIF but missing in TSF

◗ : bands that are found in TSF but missing in TIF


TIF
TSF
C
94K
67K
43K
30K
20K
14K

DISCUSSION

DISCUSSION

The membranes of several Gram-negative bacteria have been isolated, separated and characterized. Several separation methods have been used depending on the bacteria and the ultimate aims of the studies. Cell wall and cell membrane of enteric bacteria such as: E. coli and Salmonella typhimurium were studied in detail and it appears that numerous cell disintegration methods can be used. The outer and cytoplasmic membranes can be separated successfully by sucrose gradient centrifugation or by differential solubility in nonionic detergent. The study of membrane of gliding bacteria has been very limited and the work of Kleining (1972) on Myxococcus fulvus. Irschik and Reichenback (1977) on Flexibacter elegans; as well as Orndorff and Dworkin (1980) on Myxococcus xanthus indicate that the isolation of membranes from gliding bacteria is difficult.

Four different methods for isolating the outer and cytoplasmic membrane of M. xanthus A^+S^+ were attempted in the course of study. Crude membranes isolated by Method I were resolved into cytoplasmic and outer membranes by sucrose gradient centrifugation. The density of cytoplasmic membranes (Fraction I) was 1.209 g/cc and outer membrane (Fraction III) was 1.148 g/cc. Fraction II appeared to be unresolved or hybrid complex of outer and cytoplasmic membrane and had a buoyant density of 1.170 g/cc. That the outer membrane is less dense than the cytoplasmic membrane, is opposite to the membranes in enteric organisms. The outer membrane of enteric

bacteria are denser (approximately 1.22 g/cc) than cytoplasmic membrane (approximately 1.14 g/cc). However, similar results were obtained by Irschik and Reichenbach (1977) and Orndorff (1980). Orndorff found that the density of outer membrane of M. xanthus was 1.166 g/cc and that of cytoplasmic membrane was 1.22 g/cc, which are very similar to our finding. Orndorff suggested the low buoyant density of the outer membrane resulted from the unusual high phospholipid content in the outer membrane. The high density of the cytoplasmic membrane might be a result of the hydrophilic pigment associated with cytoplasmic membranes. The difference in distribution of LPS, NADH oxidase activity and succinate dehydrogenase activity were significantly different in outer and cytoplasmic membranes. The result clearly demonstrated that the heavier fraction was the cytoplasmic membrane and the less dense fraction was the outer membrane, a conclusion agrees with that of Orndorff and Dworkin (1980). There are only minor difference between the results from Method I and that of Orndorff and Dworkin (1980). These differences might be attributed to differences in the method of cell disintegration. The SDS polyacrylamide gel electrophoresis pattern did not show significant differences between the two membrane fractions in contrast to those of other gram-negative. This problem was also encountered by Orndorff and Dworkin (1980). SDS polyacrylamide gel electrophoresis pattern of the outer membrane of the wild type (A^+S^+) and the three mutants (A^+S^-, A^-S^+, A^-S^-) also did not show any significant difference. This was surprising

because we expected that motility behavior of M. xanthus to be associated with the outer membrane. The electrophoretic profiles of outer membrane proteins of cells engaged in fruiting body formation was encouraging because there were differences that suggested the synthesis of new proteins during this process.

In the second method different fractions of membranes were obtained by lysing spheroplasts with detergent. It was found that glycerol (instead of sucrose) had to be used, to stabilize spheroplasts formed by lysozyme treatment. The same was true when spheroplasts were prepared from the cytophaga, Flexibacter elegans (Irschik and Reichenback, 1973). Orndorff and Dworkin (1980) were able to obtain spheroplasts by lysozyme treatment of M. xanthus in 0.75 M sucrose, but the process required prolonged (12 hours) incubation with EDTA and Lysozyme. When spheroplasts were lysed by sonication, three fractions were obtained after sucrose gradient centrifugation. The buoyant densities were similar to those of fractions obtained by Method I, but the distribution of enzymes activity and LPS showed that the denser fraction was outer membrane while the less dense was cytoplasmic membrane. This contradicted the results obtained by Method I. When sonication was omitted, the results were similar to those obtained by Method I. The difference in results is likely due to sonication which leads to extensive redistribution of protein between membranes (Schnaitman 1981). Isolation of membranes by Method II led to formation of an intermediate,

presumably hybrid fraction that contained significant amounts of enzyme activities and LPS. Also fractions isolated by sucrose gradient centrifugation dissociated into several fractions when recentrifuged on sucrose gradients. These observations place some doubt on the effectiveness of Method II for purifying outer and cytoplasmic membranes.

Method III was based on several methods. The method of Mizuno and Kageyama (1978) was modified since sedimentation of spheroplasts by centrifugation resulted in an alternation such that they could not lysed by osmotic shock or detergent. A heavy fraction (RH) isolated from supernatant after centrifugation of spheroplasts exhibited outer membrane characteristics. It had a density of 1.192 g/cc and contained twice the amount of LPS than the cytoplasmic membrane (Table 5). The lighter fraction (RL); obtained from spheroplasts lysed in the French press, showed cytoplasmic membrane characteristics. It had a density of 1.154 g/ml and contained five times more NADH oxidase activity than RH faction (Table 6). These results contradict those obtained by Method I. Once again the middle fraction fraction (RM) showed a significant amount of outer and cytoplasmic membrane properties.

The electrophoretic profiles of RH and RL (Fig. 16) showed that there were 6 bands missing from the outer membrane fraction that were present in cytoplasmic membrane fraction (RL). The outer membrane had 2 bands that were missing from cytoplasmic membrane fraction. The electrophoretic profile of RM fraction showed that it was a combination of

RH and RL fractions indicating that it was a hybrid of outer and cytoplasmic membranes.

Method IV takes advantage of the differential solubility of different membrane fractions in Triton X-100. This method yielded membrane fractions that showed differences in protein composition as judged by the SDS-polyacrylamide gel electrophoresis (Fig. 17). The results shown in Tables 7 and 8 clearly indicate that the outer membrane, which contains three times more LPS, was present exclusively in the Triton X-100-insoluble fraction while the Triton X-100-soluble fraction, which contains more succinate dehydrogenase activity, was composed primarily of cytoplasmic membrane.

Method III is the best method for identifying outer membrane or outer membrane protein because it shows good homogeneity among the purified membranes, differences in both chemical and enzymatic characteristic between the outer and cytoplasmic membrane and it also shows difference in the electrophoretic profile between different fractions. Method I is unable to show distinct difference in the electrophoretic profile between the outer and cytoplasmic membranes. Method II lacks homogenicity among the purified membrane fractions. Method IV involves a very time consuming procedure.

When compared to Orndorff and Dworkin method, Method III has the advantages of being a shorter and less time consuming method. It does not treat the cells as harsh as the method by Orndorff and Dworkin. It also show more distinct differences between outer and cytoplasmic membrane in the electrophoretic profile.

This method for isolating and identifying outer membrane can be applied to developing cells and several aggregation deficient mutants which would then lead us to examine the role of some of the membrane proteins during cell aggregation or development.

REFERENCES

REFERENCES

Bassford, P. J., Jr., Diedrich, D. L., Schnaitman, C. L., & Reeves, P., 1977. Outer Membrance Proteins of E. coli VI. Protein alteration in Bacteriophage Resistance mutants. J. Bact. 131 : 608-622.

Bayer, M. E., Koplow, J., & Goldfine, H., 1975. Alternation in envelope structure of Heptose-deficient mutants of E. coli as revealed by freeze etching. P.N.A.S. 72 : 5145-5149.

Bishop, D. G., Work, E., 1964. An Extracellular Glyocolipid produced by E. coli grown under Lysine-limiting conditions. Biochem. J. 96 : 567-576.

Braun, V., Hantke, K., 1977. In "Microbral Interactions", ed. J. L. Reissig. London : Chapman & Hall In Press.

Braun, V., & Rehn, K., 1969. Chemical Characterization Spatial Distribution and Function of a Lipoprotein (Murein-Lipoprotein) of E. coli Cell Wall. Eur. J. Biochem. 10 : 426-438.

Bretscher, A. & Kaiser, D., 1977. Nutrition of Myxococcus xanthus, a Fruiting Myxobacterium. J. of B. 133 : 763-768.

Campos, J. M. & Zusman, D. R., 1975. Regulation of development in Myxococcus xanthus : Effect of 3 : 5-Cyclic AMP, ADP, and nutrition. P. N. A. S. 72 : 518-522.

Cronan, J. E., 1979. In "Bacterial Outer Membrances, Biogenesis and Functions", ed. Masayori Inouye. John Wiley & Son Inc. New York, pp. 35-67.

DePamphilis, M. L. & Adler, J., 1971. Attachment of Flagellar Basal Bodies to the Cell Envelope : Specific Attachment to the outer, Lipopolyasaccharide Membrane and the Cytoplasmic Membrane. J. of Bact. 105 : 396-407.

DiRienzo, J. M., Nakamura, K., Inouye, M., 1978. The outer membrane proteins of Gram-negative Bacteria : Biosynthesis, Assembly, and Functions. Ann. Rev. Biochem. 47 : 481-532.

Dworkin, M., 1962. Nutrition Requirement for Vegetative Growth of M. xanthus. J. of Bact. 84 : 250-257.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H., 1971. Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocytes Membrane. Biochemistry. 10 : 2606-2617.

Foster, H. A., & Parish, H. J., 1973. Ribosomes ribosmal subunits and ribosomal proteins from M. xanthus. J. of General Microbiology. 75 : 391-400.

Fox, C. F., Law, J. H., Tsukagosh, N., Wilson, G., 1970. A Density Label for Membranes. P. N. A. S. 67 : 598-605.

Hemphill, H. and Zahler, S., 1968. Nutritional induction and

suppression of fruiting in Myxococcus xanthus. J. of Bact. 95 : 1018-1023.

Ichihara, S., & Mizushima, S., 1977. Involvement of outer membrane protein in Enterochelin-mediated Iron uptake in E. coli. J. of Biochemistry. 81 : 746-749.

Inouye, M., Inouye, D., Zusman, D. R., 1979. Gene expression during development of Myxococcus xanthus : Pattern of protein synthesis. Developmental Biology. 68 : 579-591.

Inouye, M., Shaw, J., and Shen, C., 1972. The assembly of a structural Lipoprotein in the Envelope of E. coli. J. Biol. Chem. 247 : 8154-8159.

Irschik, H. and Reichenbach, H., 1978. Intracellular Location of Flexirubins in Flexibacter elegans (Cytophagales). Biochimica et. Bioplysica Acta. 510 : 1-10.

James, R., 1975. Identification of a outer membrane protein of E. coli, with a role in the coordination of Deoxyribonucleic acid replication and cell enlongation. J. of Bact. 124 : 918-929.

Janda, J. & Work, E., 1971. A Colorimetric Estimation of Lipopolysaccharides. FEBS Letters. 16 : 343-345.

Johnson, R. Y., White, D., 1972. Myxospore formation in Myxococcus xanthus : Chemical charges in the cell wall during cellular morphogenesis. J. of Bact. 112 : 849-855.

Johnson, R. Y., White, D., 1968. Peptidoglycan of Myxococcus xanthus. : Structure and Relation to Morphogenesis. J. of Bact. 95 : 2186-2197.

Kaiser, D., Manoil, C., Dworkin, M., 1979. Myxobacteria : Cell Interaction, Genetics, and Development. Ann. Rev. Microbiol. 33 : 595-639.

Kamio, Y., Nikaido, H., 1976. Outer Membrane of Salmonella typhimurium : Accessibility of Phospholipid Head Groups to Phospholipase C and Cyanogen Bromide Activated Dextran in the External Medium. Biochemistry. 15 : 2561-2570.

Kamio, Y., Nikaido, H., 1977. Outer Membrane of Salmonella typhimurium. Indentification of protein exposed on cell surface. Biochim. Biophys. Acta. 464 : 589-601.

Kleinig, H., 1972. Membrane from Myxococcus fulvus (Myxobacterales) containing Carotenoid Glucosides. Biochim. Biophys. Acta. 274 : 489-498.

Koplow, J. & Goldfine, H., 1974. Alternations in the outer membrane of the cell envelope of Heptose-Deficient Mutants of E. coli. J. of Bact. 117 : 527-543.

Kottel, R., Bacon, K., Clutter, D., & White, D., 1975. Coats from Myxococcus xanthus : Characterization and synthesis during myoxospore differentation. J. of Bact. 124 : 550-557.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London). 227 : 680-685.

Layne, E., 1957. In "Methods in Enzymology", ed. by Colowick, S. P. & Kaplan, N. O. Vol. 3. Academic Press. New York. P. 447-457.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin Phenol reagent. J. Biol Chem. 193 : 265-275.

Manning, P. A., Puspurs, A., Reeves, P., 1976. Outer membrane of E. coli K-12 : Isolation of mutants with altered protein 3A by using Host Range Mutants of Bacteriophage K3. J. of Bact. 127 : 1080-1084.

Manning, P. A., 1979. In "Bacterial Outer Membranes. Biogenesis and Functions." ed. Masayori Inouye. John Wiley & Son Inc. New York, pp. 409-449.

McCurdy, D. H., 1974. In "Bergey's Manual of Determinative Bacteriology". P. 76-98. Ed. Cowan, S. T., Holt, J. G., Liston, J., Murray, R. E., Niven, C. F., Ravin, A. W., and Stanier, R. Y. The Williams & Wilkins Co., Baltimore.

Mirelman, D., 1979. In "Bacterial Outer Membranes; Biogenesis and Functions". Ed. Masayori Inouye. John Wiley & Son Inc. New York, pp. 115-167.

Miura, T., Mizushima, S., 1969. Separation and properties of outer and cytoplasmic membranes in E. coli. Biochim. Biophys. Acta. 193 : 268-276.

Mizuno, T. & Kageyama, M., 1978. Separation and characterization of the outer membrane of Pseudomonas aeruginosa. J. Biochem. 84 : 179-191.

Mizushima, S. and Yamada, H., 1975. Isolation and characterization of two outer membrane preparations from E. coli. Biochim. Biophys. Acta. 375 : 44-53.

Morrison, C. E., Zusman, D. R., 1979. Myxococcus xanthus mutants with temperature-sensitive, stage-specific defects : Evidence for independent pathways in development. J. of Bact. 140 : 1036-1042.

Nakae, T., 1976. Identification of the outer membrane protein of E. coli that produces Transmembrane Channels in Reconsituted Vesicle Membrane. Biochim. Biophys. Res. Commun. 71 : 877-884.

Nakamura, K., & Mizushima, S., 1976. Effect of heating in Dodecyl-Sulfate solution on the conformation of electropheretic mobility of isolated major outer membrane protein from E. coli. J. Biochem. 80 : 1411-1422.

Nikaido, H., Takeuchi, Y., Ohnishi, S., Nakae, T., 1976. Outer membrane of Salmonella typhimurium. Electron Spin

Resonance studies. Biochim. Biophys. Acta. 465 : 152-164.

Orndorff, P. E. and Dworkin, M., 1980. Separation and properties of the cytoplasmic and outer membranes of vegetative cells of Myxococcus xanthus. J. of Bact. 141 : 914-927.

Orlowski, M., Martin, P., White, D. & Wong, M. C. W., 1972. Changes in activity of Glyoxyolate cycle enzymes during Myxospore Development in M. xanthus. J. of Bact. 111 : 784-790.

Orlowski, M., White, D., 1974. Inactivation of Isocitrate Lyase during Myxospore Development in M. xanthus. J. of Bact. 118 : 96-102.

Osborn, M. J., Gander, J. E., Parisi, E. & Carson, J., 1972. Mechanism of assembly of the outer membrane of Salmonella typhimurium. J. of Biological Chemistry. 247 : 3962-3972.

Osborn, M. J., 1979. In "Bacterial Outer Membranes Biogenesis and Functions." Ed. Masayori Inouye. John Wiley & Son Inc. New York, pp. 15-35.

Rogers, W. E., Gilleland, H. E. Jr. & Eagon, R. G., 1969. Characterization of a protein-Lipopolysaccharide complex released from cell wall of Pseudomonas aeruginosa by ethylenediaminetetraacetic acid. Can. J. Microbiol. 15 : 743-748.

Rosenberg, E. M., Katarski, M. and Gottlieb, P., 1967. Deoxyribonucleic acid synthesis during exponential growth and microcyst formation in Myxococcus xanthus. J. of Bact. 93 : 1402-1408.

Rosenberg, E., Filer, D., Zafriti, D., Kindler, S. H., 1973. Aspartokinase activity and the developmental cycle of Myxococcus xanthus. J. of Bact. 115 : 29-34.

Rosenbush, J. P., 1974. Characterization of the major envelope protein from E. coli. J. Biol. Chem. 249 : 8019-8029.

Rothfield, L. and Pearlman-Kothenez, M., 1969. Synthesis and assembly of bacterial membrane components. J. Mol. Biol. 44 : 477-492.

Sadler, W. and Dworkin, M. 1966. Induction of cellular morphogenesis in Myxococcus xanthus II. Macromolecular synthesis and mechanism of inducer action. J. of Bact. 91 : 1520-1525.

Schnaitman, C. A., 1970[a]. Examination of the protein composition of the cell envelope of Escherichia coli by polyacrylamide gel electrophoresis. J. of Bact. 104 : 882-889.

Schnaitman, C. A., 1970[b]. Protein composition of cell wall and cytoplasmic membrane of Escherichia coli. J. of Bact. 104 : 890-901.

Schnaitman, C. A., 1971[a]. Solubilization of the cytoplasmic membrane of Escherichia coli by Triton X-100. J. of Bact. 108 : 545-552.

Schnaitman, C. A., 1971[b]. Effect of Ethylenediaminetetraacetic Acid, Triton X-100, and Lysozyme on the morphology and chemical composition of isolated cell walls of Escherichia coli. J. of Bact. 108 : 553-563.

Schnaitman, C. A., 1974. Outer membrane proteins of Escherichia coli IV. Difference in outer membrane proteins due to strain and cultural differences. J. of Bact. 118 : 454-464.

Schnaitman, C. A., 1981. In "Manual of Methods for General Bacteriology." Ed. by Gevhardt, P., Editor & Chief. American Society for Microbiology. Washington, D. C., pp. 52-61.

Smit, J., Kamio, Y., Nikaido, H., 1975. Outer membrane of Salmonella typhimurium : Chemical analysis and Freeze-Fracture studies with Lipopolysaccharide mutants. J. of Bact. 124 : 942-958.

Sudo, S. and Dworkin, M., 1968. Resistance of vegetative cells and microcysts of Myxococcus xanthus. J. of Bact. 98 : 883-887.

Thaxter, R., 1892. On the Myxobacteraceae, a new order of Schizomycetes. Botanical Gazette. 17 : 389-404.

Tsukagoshi, N. and Fox, F., 1971. Hybridization of membranes by Sonic Irradiation. Biochemistry. 17 : 3309-3312.

Van Alphen, W., Lugtenberg, B., Van Boxtel, R., and Rerhoef, K., 1977. Architecture of the outer membrane of E. coli K12. Biochim. Biophys. Acta. 466 : 257-268.

Weissbach, A. and Hurwitz, J., 1959. The formation of 2-Keto-3 deoxyheptonic acid in extracts of Escherichia coli B1 identification. J. Biol. Chem. 234 : 705-709.

White, D., Dworkin, M., and Tipper, D. J., 1968. Peptidoglycan of Myxococcus xanthus : structure and relation to morphogenesis. J. of Bact. 95 : 2186-2197.

White, D., Lennarz, W. J. and Schnaitman, C. A., 1972. Distribution of lipids in the wall and cytoplasmic membrane subfractions of the cell envelope of E. coli. J. of Bact. 109 : 686-690.

White, D., 1975. "Myxospores of Myxococcus xanthus. Spore VI" Ed. by Gerhardt, Sadoff and Costilow. American Society for Microbiology, Washington, D. C. In Press.

Wireman, J. W. and Dworkin, M., 1975. Morphogenesis and developmental interactions in Myxobacteria. Science. 189 : 516-523.

Yajko, D. M. and Zusman, D. R., 1978. Changes in cyclic AMP levels during development in M. xanthus. J. of Bact. 133 : 1540-1542.