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BIOCHEMICAL, MORPHOLOGICAL, AND PHYSIOLOGICAL STUDIES ON THE METHANE OXIDIZING BACTERIUM, METHYLOSINUS TRICHOSPORIUM, WITH EMPHASIS ON STRUCTURE, FUNCTION, AND COMPOSITION OF THE INTRACYTOPLASMIC MEMBRANES.

The Ohio State University, Ph.D., 1973
Microbiology

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BIOCHEMICAL, MORPHOLOGICAL, AND PHYSIOLOGICAL STUDIES ON THE METHANE OXIDIZING BACTERIUM, METHYLOSINUS TRICHOSPORIUM, WITH EMPHASIS ON STRUCTURE, FUNCTION, AND COMPOSITION OF THE INTRACYTOPLASMIC MEMBRANES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Terry Lee Weaver, B.S.,M.S.

* * * * *

The Ohio State University
1973

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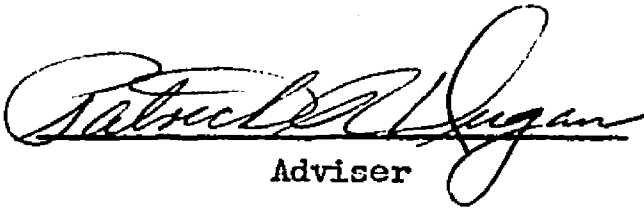

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"Men love to wonder, and that is the seed of our science".

R. W. Emerson (1870)

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INTRODUCTION

It is only recently that the methane oxidizing bacteria have been subjected to rigorous investigation. Researchers are beginning to realize the ecological significance of this group of microorganisms. However, certain pertinent areas of research have been neglected or treated only lightly. For example, one of the most distinguishing features of the methane oxidizing bacteria is the presence of systems of intracytoplasmic membranes, yet, investigations into membrane physiology and biochemistry are practically nonexistent. Morphology of the intracytoplasmic membranes has received somewhat more attention, but again, this has been rather restricted. Only thin sections have been used to study these membranes, but no one has published serial sections of these bacteria. Therefore, even though morphological membrane studies were more popular than physiological and biochemical membrane research, there were deficiencies in this area also. While it was the ecological significance of the methane oxidizing bacteria that originally attracted this author, interest was further whetted by the virgin areas open to research particularly in the area of membrane structure and function. It was from such ideas that these studies originated.

STATEMENT OF THE PROBLEM

The purpose of this work was to examine the physiology, morphology, and growth of the methane oxidizer, Methylosinus trichosporium, with particular emphasis directed at elucidating the structure, composition, and function of the intracytoplasmic membranes of this bacterium and their relationship to the methane oxidation process.

REVIEW OF THE LITERATURE

Isolation, Cultivation, and Distribution of Methane Oxidizing Bacteria

Bacterial methane oxidation was first reported in a brief communication by Kaserer (1905) although he did not isolate the microorganism involved. Nearly simultaneously came a similar report from the Delft School of Microbiology by Söhngen (1906). He was studying the oxidation of the lower hydrocarbons including methane and isolated the methane oxidizing cultures from canal water. Methane oxidation was observed in a liquid culture containing $MgNH_4PO_4$, K_2HPO_4, NH_4Cl, and $CaSO_4$, pH 6.8, incubated under an atmosphere of methane and air. Söhngen employed a rather interesting culturing device in his work. That is, he used a culture vessel containing the bacteria and the previously described medium under methane and air which was connected by glass tubing to a vented reservoir vessel containing only the mineral salts medium. As the bacteria in the culture vessel removed methane and oxygen from the head space of the culture vessel, a negative pressure resulted in the vessel. This pressure differential forced fresh liquid medium from the reservoir into the culture vessel. Söhngen could not only quantitate methane oxidation by observing

movement of the culture medium between the two vessels, but he also had a convenient semi-continuous culturing system. Isolation of pure cultures was achieved by inoculating these culture units with serially diluted enrichment cultures. Using similar culture techniques, Giglioni and Masoni (1914) isolated methane oxidizing bacteria from a variety of soils.

At this point in time, methane oxidizing bacteria were considered microbial oddities and very little was done with them until Hutton and Zobell (1949) isolated pure cultures from marine sediments. The isolation of methane oxidizing bacteria from the marine environment coupled with their previous isolation from soil and fresh water emphasized the ubiquity of these microorganisms. A phosphate buffered mineral salts medium containing $CaCO_3$, $MgSO_4$, and NH_4Cl was used in this work (Hutton and Zobell,1952). These investigators obtained methane oxidizing cultures from marine sediments using a modified Söhngen apparatus and then isolated pure cultures by streaking on medium solidified with either washed agar or silicia gel. Only about 2% of the colonies picked from washed agar proved to be methane oxidizers while 18% of those isolated on silicia gel could oxidize methane. Attempts to isolate pure cultures on nutrient agar were unsuccessful.

Strawinski and Tortorich (1955) while working with soil cultures in a mineral salts-hydrocarbon gas-air system found that the

addition of 0.02% Tween 80 prevented the characteristic clumping of these microorganisms. This ability to prevent clumping greatly facilitated the isolation of pure cultures on silicia gel medium.

Dworkin and Foster (1956) isolated methane oxidizing bacteria from the leaves of the aquatic plant Elodea obtained from a shallow fresh water pond. Isolation of cultures was carried out in cotton stoppered test tubes or in small Erlenmeyer flasks in ten liter desiccators filled with methane and air. A mineral salts medium much more complex than previously reported was used in these studies. Nitrate was employed as a nitrogen source and trace metals including iron, copper, manganese, molybdenum, zinc, boron, and magnesium were added to the medium. Additionally, agar extract and pantothenic acid were added to the medium for use as a liquid broth but were deleted when solidified with agar.

Leadbetter and Foster (1958) used a complex mineral salts medium similar to that of Dworkin and Foster minus the pantothenic acid and agar extract. The medium was solidified with 2% Bacto-Agar. Isolations were achieved by streaking soil, pond mud, sewage, or aquatic plant enrichments onto petri plates of the above medium and incubating under an atmosphere of methane and air (1:1) in a desiccator. These workers were also able to isolate methane oxidizing bacteria directly from soil by sprinkling soil particles over the surface of agar plates and incubating as

described in the preceding sentence. Incubation was at 30 C. or 37 C. and resulted in different isolates depending upon the temperature of incubation. Different isolates were also observed depending upon whether the culture was shaken or stationary. These various isolates will be described in the next section. Later studies by Foster and Davis (1966) used 50 C. incubation to select specifically for a methane-dependent coccus.

Brown et al. (1964) isolated pure cultures of methane oxidizing bacteria by inoculating serial dilutions of either soil or mud into Söhngen units, followed by reinoculation of the various dilutions back into Söhngen units. Additionally, these investigators introduced the technique of maintaining cultures in stoppered serum bottles and injecting methane with a syringe through the stopper into the bottle.

Davis et al. (1964) isolated nitrogen fixing methane oxidizing bacteria via standard streak plate technique on a nitrogen free mineral salts medium solidified with washed agar.

Recently, Whittenbury (1969) made some observations that were very helpful in subsequent pure culture isolation attempts. He tried a variety of techniques for the isolation of pure cultures of methane oxidizing bacteria and finally decided the more simple the procedure, the more successful the results. After obtaining enrichments, he diluted them to 10^{-6} and spread them directly on plates of mineral salts agar instead of transferring them to fresh

media as was then the common practice. He observed that better results were obtained by using spread plates than by using streaked plates. This was found to be due to movement of protozoa along the streaks which devoured bacteria and caused widespread contamination. On spread plates, protozoan activity was low and did not hinder subsequent isolations. Another important observation was that the organisms growing at the highest dilutions rarely proved to be methane oxidizers. In fact, methane oxidizers usually appeared at two to three dilutions lower than those containing non-methane oxidizers. Microscopic examination of methane oxidizing colonies revealed them to be very dense in appearance, and of such dense colonies picked and grown, over 90% proved to be methane oxidizers. Additionally, pure culture isolation was much more successful when the colonies were small, i.e. about 0.1 millimeters in diameter. Using these techniques, Whittenbury was successful in obtaining over 70 pure isolates of methane oxidizing bacteria. Several representative isolates will be described later.

The Methane Oxidizing Bacteria

Biological methane oxidation at the time of this writing seems to be restricted to procaryotic microorganisms. More specifically, the microorganisms oxidizing methane with two reported exceptions are Gram negative bacteria. One exception is the isolation by

Nechaeva (1949) of a mycobacterium capable of oxidizing methane. Another exception is a report by Perry (1968) of a Gram positive, nonmotile, rod shaped bacterium tentatively identified as Brevibacterium which was capable of methane oxidation. The discovery of methane oxidation by Gram positive bacteria indicates that the process is not restricted to Gram negative bacteria and suggests that possibly by using different isolation procedures, more Gram positive methane oxidizing bacteria could be found.

The remainder of the methane oxidizing bacteria that have been isolated and described are all Gram negative. The original isolate described by Söhngen (1906) was a rod shaped bacterium that he named Bacillus methanicus. The organism was an aquatic isolate and formed a pink pellicle in a liquid medium at room temperature. The name of this bacterium was changed to Methanomonas methanica by Orla-Jensen (1909) and then to Pseudomonas methanica by Dworkin and Foster (1956). The rods were about 0.4 micrometers wide and 1-1.5 micrometers long, usually occurring singly, but sometimes in chains up to four cells in length. The bacteria often stained unevenly giving the cells a mottled appearance. Cells were motile, possessing a single polar flagellum. On agar media the colonies were raised, whole, circular, convex, smooth, and pink in color. The pinkness tended to be

more concentrated in the central portion of the colony. The pigment was insoluble in water, but soluble in hexane, chloroform and ether, and had properties similar to cartenoids. The colonies had a mucoid appearance and tacky consistency and after one to two weeks, became very difficult to disrupt with a needle. Growth in liquid media shaken during incubation was dispersed or clumpy while unshaken cultures characteristically formed a reddish pink pellicle. Dworkin and Foster found that agar extract greatly stimulated the growth of their isolate in liquid medium. Phosphate at 0.002 M was optimal, and nitrate was found to be the best nitrogen source for growth. Ammonia was utilized very poorly while nitrate and asparagine were not utilized. Growth was best between pH 6.6 and 8.0. Later studies by Johnson and Temple (1962) revealed good growth up to pH 9.0. Optimum oxygen concentration was found to be 15% while atmospheric concentrations seem to be inhibitory. Methane concentrations from 10-90% resulted in equally good growth. Methanol was the only other carbon source that was shown to be utilized by this isolate. Leadbetter and Foster (1958, 1960) provided additional insight into the peculiarities of this organism in studies with their Pseudomonas methanica isolate. The investigators observed a $CH_4:O_2:CO_2$ ratio of 1: 0.4: 0.21 for the gaseous substrate and product balance with their isolate. They found that although their

organism could not use ethane or propane as its sole carbon substrate; it could oxidize either gas in the presence of methane to a variety of partially oxidized end products. These investigators found that stationary incubation at 30 C. of an aquatic enrichment invariably resulted in enriching Pseudomonas methanica. However, under the same conditions with a soil inoculum, a thin grayish pellicle was produced which resulted in pure cultures of nonpigmented bacteria. When cultures were incubated at 37 C., nonviscous, wrinkled, tan to chocolate pellicles developed, and only brown or nonpigmented methane oxidizers could be isolated from them. Plates sprinkled directly with soil and incubated at 30 C. yielded yellow to reddish-orange, brown, and nonpigmented isolates while those incubated at 37 C. resulted in brown and nonpigmented colonies. All these isolates were Gram negative rods about 0.6 micrometers by 1.0 micrometer and were motile by a single polar flagellum. No spore formation was observed. Leadbetter and Foster proposed that all their isolates be classified as varities of the type Pseudomonas methanica as described by Söhngen. The pigmentation, though fairly stable for a given isolate, could be seen to change occasionally, presumably by mutation, which lent support to their proposed classification scheme.

A new and different type of methane oxidizing bacterium

isolated by Strawinski and Brown (1957). These bacteria were 1.5 to 3.0 micrometers in length, 1.0 micrometer wide, nonpigmented, and motile by a single polar flagellum. On mineral salts agar, the organism would form only microcolonies i.e. 0.05-0.1 millimeters. Optimal temperature was 30 C. and optimal pH was 6.1. Methanol was the only carbon source other than methane shown to support growth. Gaseous substrate and product ratios in the cultures were $CH_4:O_2:CO_2$ (10:12:1) with nitrate as the nitrogen source. Methanol, formaldehyde, formate could be oxidized but a wide variety of organic alkanes, acids, aldehydes, and alcohols could not. Further studies with this isolate were carried out by Stocks and McCleskey (1964). Additions of organic compounds (i.e. vitamins, amino acids, peptones, and carbohydrates) did not increase colony size. Several new isolates were obtained that formed rosettes and which were antigenically similar to the Strawinski and Brown isolate. No antigenic relationship could be found, however, between these methane oxidizers and rosette forming species of Agrobacterium and Rhizobium. The authors indicated that they thought there were enough similarities to place this organism in the same genus as Söhngen's isolate although a different species. Subsequent biochemical studies concerning the mechanism of methane incorporation that will be explained later indicated that this was an inaccurate assessment.

Another type species that was quite different from any isolate at that time was the Gram negative methane dependent coccus reported by Foster and Davis (1966). On mineral salts agar the colonies were about 1 millimeter in diameter, colorless to ivory, smooth, rounded, and even bordered. The bacteria were cocci about 1 micrometer in diameter usually existing in a diplococcoid arrangement. The cells were found to be surrounded by a water insoluble polysaccharide capsule. The isolate had a temperature optimum at 37 C. but grew at 50 C. A variety of short chain alkanes and alcohols could be oxidized in the presence of methane but methanol was the only carbon source that replaced methane. Methane consumed: oxygen consumed: carbon dioxide produced was 5.7:3.3:1.0. Later studies by Patel and Hoare (1971) revealed nitrate to be the best nitrogen source although ammonia and most amino acids could also be used. In view of the unique properties of this organism, a new genus and species, Methylococcus capsulatus, were proposed. It was further suggested that in order to lend uniformity to the nomenclature, the prefix Methylo should henceforth be used to denote species of obligate methane utilizing bacteria. As mentioned before, Whittenbury provided many new isolates encompassing several new genera (1969, 1970). All his isolates were strictly aerobic, catalase and oxidase positive, Gram negative bacteria, capable of growth only on methane and

methanol. While ammonia could be utilized as a nitrogen source by these isolates, nitrate was preferred in most cases. Whittenbury provisionally divided his isolates into five genera, two vibroid groups Methylosinus and Methylocystis, two rod groups, Methylomonas and Methylobacter, and one coccoid group Methyloccoccus.

The genus Methylosinus contained two species: M. trichosporium and M. sporium. These are rod or pear shaped vibrio-like organisms that may form rosettes and are motile with a polar tuft of flagella. The outstanding characteristic of the genus is the formation of exospores. Although the morphology of the two species and the spores they formed differed somewhat, their formation, germination, and resistant properties were very similar. Spore formation was accomplished by budding of a spore from the end of the cell opposite the flagella tuft. Methylosinus sporium spores do not have a capsule while the spores of Methylosinus trichosporium are encapsulated. These spores were resistant to heat (i.e. survived 15 minutes at 85 C.) and desiccation (i.e. survived 18 months in absence of methane). Spore germination was dependent upon methane and required two to fifteen days.

Only one species of Methylocystis was isolated: M. parvus. This group was similar in structure and rosetting habit to the Methylosinus strains, but different from them in being nonmotile and nonsporeforming. Rather than a spore, this group formed a

"lipid cyst" that was resistant to desiccation but not to heat. This strain formed these cysts by accumulation of large amounts of poly-beta-hydroxybutyrate, and an alteration of the cell wall changing it from Gram negative to acid fast.

The proposed genus Methylomonas contained six species, i.e. methanica, albus, streptobacterium, agile, rubrum, and rosaceus. All the species were rod shaped organisms possessing a single polar flagellum. Whittenbury equated the Methylomonas methanica group with Pseudomonas methanica as described by Leadbetter and Foster (1958). The new genus designation Methylomonas is in agreement with the nomenclature proposals of Foster and Davis (1956). All the Methylomonas species formed what appears to be an immature Azotobacter like cyst. These "immature cysts" are not resistant to desiccation but will survive the absence of methane for four to five weeks while vegetative cells survive only three to four days. The cell wall structure of this resting stage appears to be intermediate between that of a vegetative cell and a mature cyst.

Five species were suggested for the proposed genus Methylobacter, i.e. chroococcum, bovis, capsulatus, and vinelandii. These organisms were similar to the large cell forming species of Azotobacter. The vinelandii species was the most common Methylobacter isolate. While none of these species could fix nitrogen, their similarities to Azotobacter extended to morphology,

slime and capsule formation, formation of desiccation resistant cysts, and the production of pigment on cyst formation. In fact, the cysts formed, by these isolates are indistinguishable in microscopic appearance from cysts of Azotobater, i.e. a distinct central portion, a clear inner wall space, and a brown outer coat or exine. Encystment was accompanied by the production of a pigment varying in color from yellow to rust to brown; nonencysted cultures remained creamy white. Mature cysts were not heat resistent but were desiccation resistent surviving at least eighteen months in the dried state in the absence of methane.

The Methyloccoccus species were capsulatus and minimus. Both were nonmotile encapsulated cocci. The two subgroups were separated on the basis of morphology. Methyloccoccus minimus formed chains while M. capsulatus usually existed as diplococci as described previously (Foster and Davis, 1966). Both species in this proposed genus formed an immature cyst resting stage as described previously.

These genera comprised the taxonomic scheme into which Whittenbury placed his seventy isolates. All previously existing isolates could fit within this scheme with the exception of Methanomonas methanoxidans. Thus, while the prefix Methano- is contrary to a uniformity in nomenclature, this generic name persists today largely because no one has suggested an alternative.

Davis et al. (1964) reported the isolation of a nitrogen fixing methane oxidizing bacterium which they named Pseudomonas methanitrificans. On mineral salts agar the colonies were white to yellow, usually smooth, glistening and entire. The cells existed as 1-2 micrometer motile rods. No pellicle was found even under static conditions and the cells contained lipid granules. In view of the fact that some of Whittenbury's Methylosinus strains reduced acetylene and were morphologically similar to P. methanitrificans, this isolate probably falls within Whittenbury's proposed genus Methylosinus.

Hazeu and Steenis (1970) isolated a methane oxidizing vibrio that was different from the vibrios of Whittenbury. Unlike most vibrios which possess a single polar flagellum, this isolate had up to five sub polar flagella. The organism was about 1 by 2 micrometers in size and could use methanol as well as methane as a sole carbon and energy source. On agar plates colonies were cream colored, round, entire, and 1-2 millimeters in diameter. Optimum temperature was 30-37 C. and optimum pH was around neutrality. The name Methylovibrio soehngenii was proposed for this bacterium.

Zobell's marine isolates of methane oxidizing bacteria were all Gram negative, nonsporeforming rods ranging in diameter from 0.6-1.0 micrometers and in length from 2.0-3.3 micrometers. The isolates were motile with one or more polar flagella. On agar

plates the colonies were usually brown and sticky. Ethane and propane also would support the growth of these bacteria. Optimum pH for one of these isolates was 6.5 and optimum temperature was 32 C. No names were proposed for any of these isolates.

A very specialized type of methane utilization was reported by Wertlieb and Vishniac (1967). They found that the photosynthetic bacterium, Rhodopseudomonas gelatinosa could use methane as a source of reducing power for carbon dioxide reduction.

Very recently, Davies (1973) isolated bacteria capable of coupling methane oxidation with nitrate reduction in the process of denitrification. Perhaps even more interesting is the fact that all these isolates turned out to be rather ordinary species of Alcaligenes and Achromobacter. These isolates constitute what might be termed facultative methane oxidizers.

Thus, contrary to original reports, the types of bacteria capable of methane utilization are numerous and diverse. A more complete and concise taxonomic organization for these bacteria will be discussed following reviews of pertinent information concerning the methane oxidation process and the ultra structure of the methane oxidizing bacteria.

The Biochemistry of Methane Utilization

The route of methane oxidation was first proposed by Dworkin

and Foster (1956) to proceed as follows:

$$CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$$

This proposal was made on the basis of manometric data with cell suspensions of Pseudomonas methanica showing oxygen uptake when the various intermediates were used as substrates. This proposed oxidative pathway was supported by Brown and Strawinski (1958) in studies employing various blocking agents during methane oxidation by Methanomonas methanoxidans and monitoring for accumulation of a given intermediate. When cells were allowed to metabolize in the presence of iodoacetate, a considerable quantity of methanol was produced. By using sodium sulphite as a trapping agent, the major portion of the methane consumed was present terminally as formaldehyde. In the absence of blocking agents, a significant amount of formic acid accumulated which was also observed when either methanol or formaldehyde was used as substrate. Therefore, the rather direct method of oxidation to carbon dioxide via methanol, formaldehyde, and formic acid stands today as the scheme for methane oxidation.

While the general pathway for methane oxidation seems fairly clear, the exact nature of the first oxidation to methanol is rather obscure. Whittenbury (1969) observed doubled cell yields

with Methanomonas methanoxidans grown on methanol as compared with growth on equimolar methane. He pointed out that the growth of this same bacterium on methane in the presence of iodoacetate as reported by Brown and Strawinski (1958) supported his data and went on to propose that the first step in methane oxidation must be energy yielding. He further suggested that the first step must involve a hydration of methane followed by an energy yielding dehydrogenation. However, separate studies by Leadbetter and Foster (1959) with Pseudomonas methanica and by Higgins and Quayle (1970) with Pseudomonas methanica and Methanomonas methanoxidans revealed that the oxygen in methanol comes from molecular oxygen rather than water. The latter investigators directly demonstrated O^{18} incorporation into methane during initial oxidation by measuring enrichment of O^{18} in accumulated methanol; H_2O^{18} was used as a control. They went on to propose a mixed function oxidase (i.e. mono-oxygenase) as the probable mechanism for this reaction. Of further interest regarding the reaction was the report by Ribbons and Michalover (1970) and Ribbons and Higgins (1971) of methane oxidation by a cell-free particulate fraction from Methyloccocus capsulatus that utilized oxygen and methane in a relationship compatible with that which would be expected for a monooxygenase. Hence, the data point to monooxygenase mediated methane oxidation coupled in some manner to

ATP production. The mechanism by which the energy is captured, however, and even the exact mechanism by which the oxidation proceeds remains unknown.

While the remainder of the methane oxidation pathway is somewhat better understood, it is by no means thoroughly elucidated. Patel and Hoare (1971) reported that the methanol oxidation in Methyloccocus capsulatus is catalyzed by a nonspecific primary alcohol dehydrogenase which was found to be independent of pyridine nucleotides and required ammonium ions for activation. A later report by Patel et al. (1972) stated that this nonspecific primary alcohol dehydrogenase had a molecular weight of 120,000 being comprised of two 60,000 monomers. The enzyme had a pH optimum of 9.0 and catalyzed the oxidation of formaldehyde as well as primary alcohols; further, no other formaldehyde dehydrogenase other than this enzyme could be demonstrated in this bacterium (Patel and Hoare, 1971). These investigators, therefore, proposed that in Methyloccocus capsulatus the same enzyme catalized methanol and formaldehyde oxidation. However, in other studies with Pseudomonas methanica, by Harrington and Kallio (1960) this was not found to be the case. Rather, they observed an alcohol dehydrogenase similar to that of Patel and Hoare except that it would not catalyze formaldehyde oxidation; a separate NAD dependent specific formaldehyde dehydrogenase was detected. In spite of this,

Patel and Hoare (1971) broadened their common enzyme proposal to include "bacteria" in general, attributing Harrington and Kallio's results to an odd isolate (i.e. the Iowa strain). Finally, in completing the pathway it should be mentioned that formate dehydrogenase activity is very common and has been frequently demonstrated in organisms utilizing one carbon compounds (Ribbons et al., 1970).

The mechanism of methane fixation into cell material is now well elucidated. The first indication of a mechanism for methane fixation was reported by Kaneda and Roxburgh (1969). These investigators observed serine as the first product in the incorporation of C^{14} methanol into cell materials by a Pseudomonas. They further proposed a serine hydroxymethylase enzyme as mediating this incorporation. This reaction proceeds as follows:

$$\begin{array}{c} 5,10 - \text{methylenetetrahydrafolate} + \text{glycine} \\ \downarrow \\ \text{serine} + \text{tetrahydrofolate} \end{array}$$

Quayle et al. (1961) in a rather extensive study with another methanol oxidizing pseudomonad, identified as Pseudomonas AM1, verified this pathway for cellular incorporation of C^{14} methanol (Large et al., 1961). They also observed that incorporation into tetrahydrofolate of the one carbon unit occurred at the level of formaldehyde

(Large and Quayle, 1963). Later studies on a mutant Pseudomonas AM1 lacking hydroxypyruvate reductase revealed that this enzyme was essential for growth on one carbon compounds (Heptinstall and Quayle, 1970, Harder and Quayle, 1971a,b). This was interpreted as an indication that the metabolism of serine formed by methanol fixation proceeded through glyceric acid via hydroxypyruvate reductase. While all this work was done with a methanol utilizing bacterium incapable of methane oxidation, other studies by Lawrence, Kemp, and Quayle (1970) showed C^{14} methanol incorporation into Methanomonas methanoxidans. This work verified the so called serine pathway as the means of one carbon fixation in Methanomonas methanoxidans. Lawrence and Quayle (1970) reported hydroxypyruvate reductase activity in cell-free extracts of Methylosinus sporium, Methylosinus trichosporium, Methylocystis parvis, and Methanomonas methanoxidans.

Proceeding chronologically parallel with much of this research on the serine pathway was another line of investigation regarding methane fixation. This particular line of research began with the observation by Johnson and Quayle (1965) that Pseudomonas methanica rapidly incorporated C^{14} methane into sugar phosphates, mainly those of fructose and glucose. They suggested that perhaps a modified pentose phosphate cycle similar to the Calvin cycle

might explain this incorporation. Therefore, another means of methane fixation very different from the serine pathway was discovered. A subsequent study by Kemp and Quayle (1967) with Pseudomonas methanica revealed that, as with the serine pathway, incorporation of the one carbon unit was at the formaldehyde level. Further, this incorporation was shown to proceed by coupling of formaldehyde to D-ribose-5-phosphate forming allulose phosphate.

D-ribose-5-phosphate + formaldehyde → allulose-6-phosphate

Lawrence et al. (1970) later reported that Methyloccocus capsulatus also fixes methane by this allulose phosphate pathway. Lawrence and Quayle (1970) also detected hexose phosphate synthelase activity in the Methylomonas and Methylobacter isolates of Whittenbury as well.

Therefore, to date, two pathways for the fixation of methane have been demonstrated, the serine pathway and the allulose phosphate pathway. Incorporation in both cases is at the level of formaldehyde. All methane oxidizing isolates examined thus far possess one of these pathways (Lawrence and Quayle, 1970). That is, they either have the serine pathway or the allulose phosphate pathway, but not both.

Biological Membranes

One of the most obvious and distinctive morphological characteristics of the methane oxidizing bacteria is the presence of complex systsms of intracytoplasmic membranes. Before proceeding with a description of these membranes, however, a very cursory examination of biological membrane literature in general should be beneficial.

The idea of a distince boundary membrane at cell surfaces originated in 1855 when Nageli and Cramer studied pigment penetration into intact and damaged cells and concluded they were bounded by a plasma membrane. They further suggested that the osmotic properties of cells were due to this membrane. On the basis of permeability studies, Overton (1895) reported that the plasma membrane contained lipids. Then Gorter and Grendel (1925) extracted the lipids from red blood cell membranes, spread the extracted lipid as monomolecular film and found the lipid occupied twice the area of the membranes. They postulated that the lipid must exist as a bilayer with the hydrophobic parts of the lipid molecules facing inward. Danielli and Davson (1936) reported that membranes also contain protein. They postulated that this protein coats both sides of the lipid bilayer of Gorter and Grendel. With the development of electron microscopy, osmium fixed membranes were seen to exist as a 10 nanometer

trilamellar unit with two dense lines separated by a region of low density; this was readily related to the lipoprotein sandwich of Danielli and Davson. Because of the seeming universality of this structure, Robertson (1959) proposed that all biological membranes have the same basic structure, the so called unit membrane theory. The Lipid-Globular Protein mosaic model of membrane structure was proposed by Lenard and Singer (1966). This model suggests that hydrophobic portions of lipids (phospholipids) and a large fraction of the nonpolar amino acid residues of the protein are sequestered away from contact with water in the hydrophobic interior of the membrane while the ionic groups of both the lipids and the proteins are in direct contact with water. The phospholipids in this mosaic model are primarily arranged in a bilayer form, but the ionic groups of the lipids are exposed directly to the aqueous phase, and the bilayer is not continuous. Recently, Singer and Nicolson (1972) revised this concept to the fluid mosaic model of membrane structure. This theory describes membranes to exist basically as lipid structures wherein amphipathic proteins are found floating in fluid liquid matrix. This model has strong appeal in that it is thermodynamically sound and can explain certain phenonema that no other model explains. For example, membrane antigens can be observed to migrate to a uniform distribution over the new cell surface within a short time after two antigenically different cells

fuse together. More recently, Singer (1973) sited numerous reports published since he proposed the fluid mosaic model in 1972 establishing the mobility of molecular components in cell membranes. Thus, while our knowledge has increased concerning cell membranes, the newest ideas are not drastically different from the earlier proposals of Gorter and Grendel and Danielli and Davson. Membranes play many varied rolls in biological systems. As was obvious very early but nevertheless remains very significant is the idea that membranes contain and wall in cell components isolating these components from the surrounding environment. Also recognized very early were the osmotic and permeability properties of cells which are regulated by membranes (Nageli and Cramer, 1972 and Overton, 1895). Membranes possess enzymes as an integral part of their structure that catalyze a variety of reactions including oxidations, reductions, electron transport, phosphorylation, active transport and synthetic reactions for cell components (Coleman, 1973).

Intracytoplasmic Membranes of the Methane Oxidizing Bacteria

The first report that methane oxidizing bacteria contained intracytoplasmic membranes was in Whittenbury's (1969) publication. He published two electron micrographs of thin sections of two of his isolates each showing a distinctive arrangement of

intracytoplasmic membranes. The first thin section described was of his Methylosinus trichosporium isolate. He wrote one sentence describing these intracytoplasmic membranes as a tubular membrane system. Since this first description involving one photograph of one thin section, this organism has not been further described in the literature with respect to its intracytoplasmic membranes. The other organism described as possessing a lamellated membrane system in this report was a Methylobacter isolate. A report by Proctor et al. (1969) soon followed in which they observed in Methylococcus capsulatus what Whittenbury had called a lamellar arrangement of intracytoplasmic membranes in his Methylobacter isolate. Additionally they described a peripheral intracytoplasmic membrane system in an unidentified methane utilizing isolate that was similar to the tubular system Whittenbury described for Methylosinus trichosporium. These investigators went on to point out the similarity between these intracytoplasmic membranes and those reported previously in photosynthetic bacteria and nitrifying bacteria; they speculated that the membranes probably play a roll in the respiratory activity of the cells. Davies and Whittenbury (1970) were the first to point out that methane oxidizing bacteria possessed one of two arrays of intracytoplasmic membranes. They either had the tubular membrane system consisting of pairs of membranes extending through the cytoplasm or near the

cell periphery or they had the lamellar system comprised of vesicular discs organized into distinct bundles. Methylococcus minimus, Methylobacter vinelandii, Methylomonas vibrum, and Methylomonas albus contained the latter type membrane which they termed Type I membrane system. Connections between the plasma membrane and the intracytoplasmic membranes of Methylomonas were reported and this is the only report of connections between these 2 membranes to date. Methylocystis parvis and Methylosinus sporium have the "tubular" type membrane arrangement which they called a Type II membrane system. Smith and Ribbons (1970) described the intracytoplasmic membranes of Methanomonas methanoxidans. They reported these membranes were arranged in concentric layers near the cell periphery and existed as flattened sacs. Chemical analysis of these membranes revealed phosphatidyl choline as the major phospholipid with an 18:1 fatty acid accounting for over 90% of the total esterified fatty acids. Smith et al. (1970) described the intracytoplasmic membranes of Methylococcus capsulatus as saccules limited by a 7.5 nanometer unit membrane arranged in stocked arrays.

Classification of Methane Oxidizing Bacteria

It has become obvious that the two main differences known to date to exist between methane oxidizing bacteria are the method of

methane fixation and the type of membrane system they possess. Apparently the methane oxidizers with a Type II membrane system all incorporate methane via the serine pathway, while those that have a Type I membrane system fix methane by the allulose phosphate pathway. With the existing data, most known isolates of obligate methane oxidizing bacteria can be place into one of these two broad groups. It should be emphasized that some of the data are sketchy and incomplete (e.g. Lawrence and Quayle, 1970, assayed for hydroxypyruvate reductase rather than serine hydroxymethyl transferase), however, the best breakdown to date would be as follows:

Group	Genus	Type Membranes	Hydroxy-pyruvate Reductase	Hexose Phosphate Synthetase
A.	Methylomonas	I	-	+
	Methylobacter	I	-	+
	Methylococcus	I	-	+
B.	Methylosinus	II	+	-
	Methylocystis	II	+	-
	Methanomonas	II	+	+/-

Significance of the Methane Oxidizing Bacteria

Within the last ten years, there has been considerable interest in the methane oxidizing bacteria as sources for protein. This interest was stimulated by the relative cost of methane gas

as compared to an equal amount of food protein (Hamer and Heden, 1967). Vary and Johnson (1967) reported yields of 70% (per quantity of methane) and generation times as low as three hours with mixed methane oxidizing cultures. For comparison, Häggstrom (1969) looked at production of microbial cells from methanol and observed 41% yield and 3.2 hours generation time. These data are in accord with that of Whittenbury (1969) where he observed about one-half the growth on methanol as compared with growth on methane. Sheehan and Johnson (1971) were able to continuously grow a mixed methane oxidizing culture obtaining 2.39 grams (dry weight) of cells per liter per hour. D'Mello (1972) examined the amino acid composition of a variety of methane utilizing bacteria to assess their potential value as food protein. He concluded that several of the strains, including Whittenbury's Methylomonas albus, had excellent compliments of the essential amino acids even ranking higher than soybean meal. However, when Wolnak et al. (1967) looked at the economics of protein production from methane, they concluded that at that time the process was too expensive to make it profitable. They did suggest , however, that due to a high vitamin content, these cells could possibly be marketed profitably as vitamin sources.

Strawinski and Tortorich (1955) mentioned the potential use

of methane oxidizing bacteria in oil prospecting.

Naguib and Overbeck (1970) attributed the main ecological significance of methane oxidizing bacteria to the rapid utilization of oxygen reserves in water. While this is surely important in some aquatic ecosystems and probably even in soil, it is a matter of opinion just what is the most significant effect the methane oxidizing bacteria have on an ecosystem. For example, Weaver and Dugan (1972) described the involvement of these bacteria as agents in carbon cycling and eutrophication in aquatic ecosystems. Additionally, methane oxidizing bacteria are now known to be capable of denitrification (Davies, 1973). This could be particularly important in soil where conditions favoring methane production also favor denitrification. Therefore, denitrification via methane oxidation may play a role that was heretofore unrecognized. The main point to be made, however, is that the methane oxidizing bacteria are ecologically significant micro-organisms for a variety of reasons.

MATERIALS AND METHODS

I. Growth of the Bacteria

A. Culture Medium

A defined mineral salts culture medium was developed by combining various components of similar published media in an attempt to minimize undesirable precipitates and labor while providing a satisfactory medium for culturing methane oxidizing bacteria. The composition of this combined medium (medium CM) is listed in Table 1. The pH of the medium was adjusted to 7.0 following which the medium was dispensed as required and autoclaved for 20 minutes at 121 C. and 15 lbs/in^2 pressure. If a solid medium was desired, 2% Difco purified agar was added prior to sterilization.

B. Methylosinus trichosporium

The OB3b strain of Methylosinus trichosporium was kindly given to me by Dr. Roger Whittenbury, University of Warwick,

Table 1. Components of mineral salts medium CM listed in grams per liter of distilled water.

KNO_3	1
$MgSO_4 \cdot 7H_2O$	2×10^{-1}
$CaCl_2$	2×10^{-2}
$FeSO_4 \cdot 7H_2O$	1×10^{-2}
Na_2HPO_4	2.3×10^{-1}
NaH_2PO_4	7×10^{-2}
$CuSO_4 \cdot 5H_2O$	5×10^{-6}
H_3BO_4	1×10^{-5}
$MnSO_4 \cdot H_2O$	7×10^{-6}
$ZnSO_4 \cdot 7H_2O$	7×10^{-5}
MoO_3	1×10^{-5}

Coventry. This is an aquatic strain isolated from lake water.

C. Maintenance of Cultures

Cultures were maintained in both liquid medium and on solid medium and could be readily transferred from one to the other. Liquid cultures were kept in rubber stoppered 74 ml serum bottles containing 25 ml CM medium. Inoculations were made by injecting culture fluid through the rubber stopper with a sterile syringe. After inoculation, 10 ml of 99.1% methane (Matheson Gas Co.) were injected into the bottles using a sterile syringe equipped with a Swinney filter. The filter contained a 0.22 micrometer pore membrane filter (Millipore) to sterilize the injected methane. The bottles were agitated on a rotary shaker at 2 cycles/sec. Cultures were also maintained on CM plates by standard techniques. The plates were incubated in desiccators filled with methane and air (1:1). Incubation of all cultures was at 21 C.

D. Isolation of Methylomonas methanica

Methylomonas methanica was isolated for comparative purposes in morphological studies. An enrichment culture was obtained by inoculating water from Lake Erie into a serum

bottle followed by injecting methane into the bottle and incubating several weeks as described previously. This culture enriched in methane oxidizing bacteria was used to inoculate culture tubes containing 10 ml of CM medium. The culture tubes were stoppered with cotton and incubated in desiccators as described previously. After several weeks, a reddish pink pellicle was obvious on the surface of the medium. A loopful of the pellicle was streaked onto CM agar plates from which isolated pink colonies could be picked. These colonies consisted of motile rod shaped bacteria that would not grow on nutrient agar. Based upon the method of isolation, the requirement for methane, and the morphology and pigmentation of the bacteria, this isolate was identified as Methylomonas methanica.

E. Growth of Large Batches of Cells

Large quantities of bacteria were grown in a Fermentation Design Model SA30-F1S 30 liter fermenter containing 15 liters of CM medium. The fermenter was modified so that the head space could be evacuated and filled with a glass wool filtered methane:air (1:1) gas mixture. The broth was stirred at one revolution per second and

incubated at 21 C. After a week, cells were harvested using a Sharples continuous flow centrifuge.

II. Growth Studies

A. Carbohydrate Utilization

Methylosinus trichosporium was tested for the ability to grow heterotrophically on 19 different carbohydrates; Bacto Differentiation Disks (Difco) were used for this purpose. For determining fermentative capacity, the disks were transferred aseptically to cotton stoppered culture tubes containing 10 ml of sterile CM medium, gas indicator tubes, and 0.04% bromcresol purple. The tubes were then inoculated with 0.2 ml of a 5 day culture and incubated at 21 C. The tubes were checked for acid and gas after 4 hours, 24 hours, and 1 week. In order to determine capacity for aerobic growth on the carbohydrates, the differential disks were aseptically transferred to plates of CM agar that had been heavily swabbed with culture. Incubation was aerobic at 21C and observation was at the above times.

B. Amino Acid Utilization

Methylosinus trichosporium was tested for ability to grow on 20 different amino acids. For this purpose,

sterile CM agar plates containing 0.5% amino acids were streaked with bacteria, and the plates were incubated aerobically or anaerobically (i.e. in CO_2 incubator at 21 C.). After 2 weeks the plates were examined for growth. Sodium salts of formate, acetate, propionate, and butyrate were tested.

C. Effects of Organic Compounds on Methane Oxidation

In view of the fact that Methylosinus trichosporium was incapable of growth on 43 organic compounds tested, several of these compounds were tested for their ability to affect methane oxidation in any manner. Serum bottles containing 10^{-2} Molar concentrations of various previously tested organic compounds in CM medium were prepared; pH was re-adjusted to 7 prior to sterilization by addition of 0.1 N HCl or 0.1 N NaOH. One ml of bacterial inoculum (Final concentration 1.8×10^7 cells/ml) was injected into each bottle. This was followed by 2 ml of 99.1% sterile methane injected to start the experiment. Incubation was at 21 C. on a rotary shaker (2 cycles/second).

D. Spectrophotometric Determination of Cell Numbers

Since these bacteria could not be enumerated by plate count, a standard curve was prepared for rapid spectro-

photometric estimations of cell numbers. Various dilutions through 10^{-4} were made of a cell suspension. The optical density of each dilution was read at 420 nm with a Gilford Model 2400 Recording Spectrophotometer and a cell count was done using a Petroff-Hauser counting chamber. Cell number was plotted against optical density to obtain a standard curve from which subsequent estimations of cell numbers could be made.

E. Growth Curve

Fifteen identical serum bottles were prepared each of which contained 25 ml CM medium and bacterial inoculum (Final concentration 1.8×10^7 bacteria/ml). Two ml of 99.1% methane were added to each bottle to start the experiment. Incubation was at 21 C. on a rotary shaker (2 cycles/second). Samples were removed at various times: at each sampling the pH was measured using a Corning pH meter and cell numbers were determined spectrophotometrically as described previously.

F. Balances of Gaseous Substrates and Products

The ratio of methane, oxygen, and nitrogen consumed to carbon dioxide produced by a cell suspension was determined.

After adding 0.1 gm (wet weight) of cells to 25 ml CM medium, 2 ml 99.1% methane were added to start the experiment. Gas samples were withdrawn periodically and were analyzed by gas chromatography. After withdrawing a sample, an equal volume of sterile medium was injected into the bottle.

G. Gas Analysis

Gas samples were analyzed using a Carle Model 8004 gas chromatograph equipped with a 100K ohm thermistor detector. For analysis of methane and/or carbon dioxide, an 8 ft X 1/8 in, 50-60 mesh silica gel column was used. For analysis of oxygen, nitrogen, and methane a 12 ft X 1/8 in, 100-120 mesh molecular sieve column was used. In both cases column temperature was 80 C and helium was the carrier gas. Carrier gas was maintained at 18 ml/min with the silica gel column and at 12 ml/min with the molecular sieve column.

III. Preparation of Isolated Intracytoplasmic Membranes

A. Cell Breakage

Bacterial cells (200mg wet weight/ml) were disrupted by shaking with 0.45-0.5 millimeter acid-washed glass beads in a Braun MSK cell homogenizer at 4000 RPM for 3 min. The homogenizer unit was continuously cooled with

liquid carbon dioxide during the process.

B. Differential Centrifugation

Various cell fractions were separated by differential centrifugation using 5/8 x 4 inch polyallomer tubes in a SW27 rotor with a Beckman Model L2-65B Ultracentrifuge. The fractionation was performed as follows:

1. Centrifuge 30 minutes at 5,000 x g.
2. Save the pellet as the 5P fraction and recentrifuge the supernatent for 30 minutes at 10,000 x g.
3. Save the pellet as the 10P fraction and recentrifuge the supernatent for 30 minutes at 20,000 x g.
4. Save the pellet as the 20P fraction and recentrifuge the supernatent for 30 minutes at 40,000 x g.
5. Save the pellet as the 40P fraction and recentrifuge the supernatent for 30 minutes at 80,000 x g.
6. Save the pellet as the 80P fraction and the supernatent as the 80S fraction.

Each fraction was examined electronmicroscopically after

negative staining with 1% (w/v) phosphotungstic acid. This procedure is outlined in Section IV. Each fraction was also analyzed for total protein, total hexose, and poly-beta-hydroxybutyrate (see Sections III C through E).

C. Protein Quantitation

Total protein in each differential centrifugation fraction was determined by a modification of the procedure of Lowry et al. (1951). The following reagents were prepared:

Reagent A.	Na_2CO_3	20 g
	NaOH	4 g
	KNaTartrate	0.2 g
	Distilled water	1000 ml
Reagent B.	$CuSO_4 \cdot 5H_2O$	0.5 g
	Distilled water	100 ml
Reagent C.	50 parts of A + 1 part B	
Reagent D.	Folin's phenol reagent	5 ml
	Distilled water	7 ml

The Folin phenol reagent was that of Folin and Ciocalteu (1927). The analysis was performed as follows:

1. Add 5 ml reagent C to a clean test tube.

2. Add 1 ml sample containing between 20 and 150 micrograms protein.
3. Begin the reaction by squirting in 0.5 ml Reagent D.
4. Mix well.
5. React 1 hour.
6. Read optical density at 660 nanometers.

Protein concentration was then determined by comparison to a standard curve prepared by reacting various concentrations of bovine albumin (Sigma Chemical Co.) as above and plotting optical density against protein concentration.

D. Hexose Quantitation

Hexoses in concentrated H_2SO_4 react with the acid to form furfural derivatives which in turn react with anthrone to form a blue-green color. Anthrone reagent contained the following:

Concentrated H_2SO_4	720 ml
Distilled H_2O	280 ml
Anthrone	500 mg
Thiourea	10 g

The analysis was prepared as follows:

1. To 1 ml sample (containing 20-200 micrograms sugar) add 5 ml cold anthrone reagent.
2. Shake well.
3. Cap and heat at 100 C. for 15 minutes.
4. Cool for 20 minutes.
5. Read optical density at 620 nanometers.

Hexose concentration was obtained by referring to a standard curve. The standard curve was prepared by analyzing various concentrations of glucose as above and plotting optical density against hexose concentration.

E. Poly-beta-hydroxybutyrate Quantitation

Poly-beta-hydroxybutyrate (PHB) was quantitated by the method of Law and Slepecky (1961). The PHB analysis of the different centrifugation fractions was performed as follows:

1. Extract the fraction with 2 ml chloroform.
2. Remove 1 ml chloroform to a clean test tube.
3. Evaporate the chloroform.
4. Add 10 ml concentrated H_2SO_4.
5. Cap the tube and heat 10 minutes at 100 C.
6. Cool.
7. Read optical density at 235 nanometers.

8. Calculate PHB using the extinction coefficient of 1.55×10^4 reported by Law and Slepecky (1961).

IV. Preparation of Specimens for Electron Microscopy

A. Freeze Etching

Freeze etching of bacteria was done as follows:

1. Concentrate cells by centrifugation.
2. Place a drop of the pellet on a gold specimen holder.
3. Freeze in liquid freon for 5 seconds.
4. Transfer quickly to liquid nitrogen.
5. Place on specimen platform cooled to -100 C.
6. Evacuate to 10^{-6} torr.
7. Fracture with knife cooled to -196 C.
8. Position the knife above specimen and etch for 4.5 minutes.
9. Shadow for 8 seconds, 110 volts, with 6 cm of 0.1 millimeters platinum wire at an angle of 32°.
10. Carbon coat for 11 seconds at 122 volts.
11. Vent to atmospheric pressure.
12. Float off the carbon replica in distilled water.
13. Transfer the carbon replica to 25% (v/v) H_2SO_4 for 15 minutes.

14. Transfer to 50% (v/v) H_2SO_4 for 15 minutes.
15. Transfer to 75% (v/v) H_2SO_4 for 15 minutes.
16. Transfer to concentrated H_2SO_4 for 2 hours.
17. Reverse the H_2SO_4 sequence to distilled water.
18. Transfer to 5% (w/v) sodium hypochlorite (Clorox) for 2 hours.
19. Wash in distilled water.
20. Pick up the carbon replica on a 300 mesh uncoated copper grid.

B. Thin Sections

The following reagents were prepared:

1. Veronal-Acetate Buffer Stock

Sodium acetate	19.428 g
Sodium barbital (Fisher)	29.428 g
Sodium chloride	34 g
Distilled water	1000 ml

2. Working concentration of Veronal-Acetate Buffer

Stock Mixture	5.0 ml
0.1N HCl	7.0 ml
1M $CaCl_2$	0.25 ml
Distilled H_2O	13.0 ml

3. 1% (w/v) OsO_4 (Mallinckrodt) in Veronal-Acetate

Buffer.

4. 5% (w/v) (J. T. Baker) Uranyl-acetate in Veronal-Acetate Buffer.

5. Mixture A:

Dodecenyl succinic anhydride (Shell)	100 ml
Epon 812 (Shell)	62 ml

6. Mixture B:

Methylnadic anhydride (Shell)	89 ml
Epon 812	100 ml

7. Embedding resin: mix thoroughly 55A to 45 B.

Fixation and embedding of samples were done as follows:

1. Add glutaraldehyde (Fisher) to the sample to a final concentration of 1%.
2. Incubate 16 hours at 5C.
3. Wash in veronal acetate buffer.
4. Fix 2 hours at room temperature in 1% (w/v) osmium tetroxide.
5. Wash in veronal acetate buffer.
6. Resuspend sample in 2% (w/v) agar and cool.
7. After agar solidifies, cut into small blocks.
8. Wash blocks in uranyl acetate for 2 hours.
9. Transfer to 30% (v/v) ethanol for 15 minutes.
10. Transfer to 50% (v/v) ethanol for 15 minutes.

11. Transfer to 70% (v/v) ethanol for 15 minutes.
12. Transfer to 80% (v/v) ethanol for 15 minutes.
13. Transfer to 95% (v/v) ethanol for 15 minutes.
14. Transfer to 95% (v/v) ethanol for 15 minutes.
15. Transfer to 100% ethanol for 15 minutes.
16. Transfer to 100% ethanol for 15 minutes.
17. Transfer to propylene oxide (Eastman Organic Chemicals) for 15 minutes.
18. Transfer to propylene oxide for 30 minutes.
19. Transfer to propylene oxide in resin (3:1) for 1 hour.
20. Transfer to propylene oxide in resin (1:1) for 1 hour.
21. Transfer to propylene oxide in resin (1:3) for 4 hours.
22. Transfer to pure embedding resin for 4-12 hours.
23. Transfer to Beem capsules filled with embedding resin.
24. Incubate capsules 12 hours at 37 C.
25. Incubate capsules 12 hours at 45 C.
26. Incubate capsules 12 hours at 60 C.
27. Blocks ready for sectioning when cool.

Thin sections were cut using either a Porter Bloom MT 1 manual ultramicrotome or a LKB Model III ultramicrotome; in both cases, freshly prepared glass knives were used.

In some cases sections were post stained with lead citrate for 5 minutes as described by Reynolds (1963).

C. Negative Staining

Samples were mixed 1:1 with 1% (w/v) phosphotungstic acid (Fisher), pH 7.0, and sprayed onto formvar coated copper electron microscope grids.

D. Electron Microscopy

Either a Zeiss EM-9 or a Phillips EM-300 electron microscope was used to examine the preparations. Photographs were taken on Kodak Electron Image glass negative plates. The plates were developed for 4 minutes (68-70 F.) in D-19 developer (Kodak) and fixed for 5-10 minutes.

V. Lipid Characterization

A. Extraction of Lipids

Lipids were extracted by modifications of the procedures of either Folch et al. (1957) or Bligh and Dyer (1959). The Folch extraction was done as follows:

1. Extract sample over night with chloroform: methanol (2:1).
2. Filter the extract through chloroform: methanol

washed Whatman #1 filter paper.

3. Wash with 1/5 volume M KCl.
4. Separate the phases by centrifugation.
5. Discard the upper aqueous phase and retain the lower lipid phase.
6. The lower layer may be washed with additional portions of Folch's upper phase (i.e. chloroform: methanol: water, 3:48:47) or methanol: water (1:1).

The Bligh and Dyer extraction was performed as follows:

1. Shake 30 ml of bacterial suspension in CM medium (overnight) with 75 ml methanol and 37.5 ml chloroform.
2. Add 37.5 ml chloroform.
3. Add 37.5 ml M KCl containing 0.4% (v/v) glacial acetic acid.
4. Shake vigorously.
5. Allow the phases to separate several hours or centrifuge.
6. Discard the upper aqueous phase and retain the lower lipid phase.

Following both extractions, the lipid preparation was concentrated using an RAE Vacuum Evaporator (RAE Motor Corp.). When preparing lipids for fatty acids studies,

all procedures were done under nitrogen.

B. Removal of Poly-beta-hydroxybutyrate (PHB)

PHB was precipitated from the lipid extract by adding 2 volumes of methanol and holding at -10 C. for several hours. The sample was then centrifuged and the PHB free lipid was recovered and concentrated as described previously. Removal of PHB was found to be necessary as a prerequisite for satisfactory separation of the various lipid components.

C. Fractionation of Lipids by Silicic Acid Column Chromatography

Silicic acid (Fisher Scientific Co.) was washed in chloroform: methanol (2:1) and heated at 110 C. for 12 hours. The following procedure was used to prepare the silicic acid column and perform the separation:

1. Mix the washed silicic acid with chloroform to form a slurry.
2. Pour the slurry into a 10 ml column.
3. Run about 25 ml of chloroform through the column to pack it.
4. Place some glass wool on top of the silicic acid to prevent mixing.

5. Layer the lipid sample (in chloroform) on top of the silicic acid.
6. Elute neutral lipids with 50 ml of chloroform.
7. Elute glycolipids with 50 ml of acetone:chloroform (1:1) and 50 ml of acetone.
8. Elute phospholipids with 50 ml methanol.
9. Collect all fractions in chloroform:methanol washed vessels.
10. Fractions concentrated as described previously.

D. Liquid/Liquid Partition Column Chromatography

Traces of water soluble components were removed from the phospholipid fraction by passage through a liquid/liquid partition column. The aqueous phase was immobilized by beads of Sephadex G-25. The procedure was as follows:

1. Soak the Sephadex G-25 (Pharmacia) overnight in 4 volumes of Folch's upper phase (FUP) prepared as previously described.
2. Wash the beads several times in FUP.
3. Pack the Sephadex slurry into a 10 ml column under slight pressure.
4. Cover the Sephadex with a piece of glass wool.
5. Run about 10 ml of FUP through the column.

6. Run about 20 ml of Folch's lower phase (FLP) through the column to displace FUP in void space.
7. Place the sample on top of the Sephadex.
8. Run about 50 ml of FLP through the column to elute the phospholipids.

E. Thin Layer Chromatography

Thin layer chromatographic plates (TLC) were prepared by shaking 40 gm of Camag silica gel type D-0 (Arthur Thomas Co.) with 85 ml water for 5-10 minutes. This slurry was then spread over clean 8 X 8 X 1/8 inch glass plates using a Desaga-Brinkman plate spreader at 0.35 millimeters. These plates were heated at 110 C for 2-4 hours prior to use. Ascending development in a solvent saturated atmosphere was the procedure always used.

For separation of the simple lipids, hexane:diethyl ether:acetic acid (70:30:1) was found to be an excellent solvent. While several different solvents were used for separation of phospholipids, chloroform:methanol:water (65:25:4) was excellent and was used for routine separations. Butanol:acetic acid:water (60:20:20) was used for comparative purposes with standards. When running phospholipids, the TLC plate was always prewashed in acetone:petroleum ether (1:3).

F. Detection of Lipids on TLC Plates

A variety of detection methods for various compounds were all used from time to time and are listed below:

1. U.V. lamp: Some lipids can be detected with ultraviolet light.
2. Phospholipid Spray Reagent: The phospholipid spray of Goswami and Frey (1971) was used; this reagent was prepared as follows:
   a. Add 250 mg ammonium molybdate to 1 ml of water and cool
   b. Add 80 mg copper metal and chill.
   c. Add 1 ml concentrated H_2SO_4; the mixture turns blue.
   d. After 2 hours of periodic shaking, add 40 ml water; the mixture turns brown.
   e. Remove excess copper.
   f. Add 3.2 ml concentrated H_2SO_4.
   g. Spray over TLC plate; phospholipid turns blue.
3. Glycolipid spray: 200 mg orcinol in 100 ml 75% (v/v) H_2SO_4. Spray TLC plate until entire surface is moist, and heat 15 minutes at 100 C. Glycolipids turn blue-violet.

4. Iodine Vapor: Neutral and phospholipids turn yellow when exposed to the vapors of iodine; glycolipids are rarely detected by this test.
5. Ninhydrin Spray: 0.2% (w/v) ninhydrin in water saturated butanol. Spray the entire area and heat at 100 C. for 15 minutes in a water saturated atmosphere. Free amino groups turn blue.
6. Dragendorf's Spray: This spray reagent, which detects choline containing phospholipids, was prepared as follows:
   a. Reagent I = 1.7 gm basic bismuth nitrate in 100 ml 20% (v/v) acetic acid.
   b. Reagent II = 40 gm potassium iodide in 100 ml water.
   c. Reagent III = Dragendorf's Reagent = 20 ml Reagent I mixed with 5 ml Reagent II and 70 ml water. This must be mixed just prior to spraying. Spots appear orange immediately or after gentle warming.
7. Plasmalogen Spray: 0.4% (w/v) dinitrophenylhydrazine in 2 N HCl. Plasmalogens appear as yellow or orange spots; warming slightly may aid color development.
8. Total Organic Spray: 70% potassium dichromate in concentrated H_2SO_4. Heat 30 minutes at 180 C. after spraying. Organic compounds turn black.

9. Hanes and Isherwood Reagent: This is a specific phospholipid spray for paper chromatograms (Hanes and Isherwood, 1949). The reagent contains 5 ml 60% (w/w) perchloric acid, 10 ml 1 N HCl, 25 ml 4% (w/v) ammonium molybdate and water to 100 ml. After spraying, dry well and heat in a water saturated atmosphere at 85 C. for 7 minutes. Remove, wait a few minutes, and expose to dilute H_2S fumes. Phospholipids turn blue.

G. Identification of Simple Lipids

Since the simple lipids were only separated into major classes (i.e. diglycerides, triglycerides, free fatty acids, and hydrocarbon) and because the TLC separation worked so well, these could be identified simply by running a representative standard for each class.

H. Identification of PHB

PHB was identified by infrared spectrophotometry. The methanol precipitate from total lipids, obtained as described previously, was used for this purpose. The sample was dissolved in chloroform, dried on a KBr pellet, and scanned through the infrared using a Perkin Elmer Model 237B recording infrared spectrophotometer. The resulting spectrum was

compared to the PHB spectra of Blackwood and Agnes (1957). Comparison was also made with a spectrum prepared using a purified PHB standard isolated in this laboratory from Zoogloea ramigera by Alice Parsons.

I. Identification of Phospholipids

Because the phospholipids were separated to individual chemical compounds and because the phospholipids were of principal interest in this study, a more rigorous means of identification was pursued.

1. Of prime importance in identification of a phospholipid was TLC along with phospholipid standards (Supelco, Inc.) in at least 2 different solvents.
2. The various spray reagents described previously were used to identify unknown compounds.
3. Paper chromatography of the water soluble mild alkaline hydrolysis products was used to identify phospholipids. Phospholipids, separated by TLC as described previously, were eluted from the TLC plate with chloroform: methanol (2:1), concentrated, and dissolved in 1 ml of chloroform:methanol (1:4). Mild alkaline hydrolysis (i.e. removal of the fatty acyl esters) was accomplished by the following procedure:

a. To the 1 ml sample add 0.1 ml 1.2 N NaOH:water (1:1).

b. Mix well and incubate at 37 C. for 10 minutes.

c. Neutralize the mixture with 1 N acetic acid.

d. Add 2 ml chloroform:methanol (9:1).

e. Add 1 ml isobutanol.

f. Add 2 ml of water.

g. Shake well and centrifuge for 10 minutes at 300Xg.

h. Draw off the upper aqueous phase with a Pasteur pipette.

i. Re-extract the lower phase 2 times with 1 ml of methanol:water (1:2).

The water soluble hydrolysis products were run on descending Whatman #1 paper chromatograms using phenol saturated water:acetic acid:ethanol (100:10:2) solvent. Phospholipids (Supelco, Inc.) treated in the same manner were used as standards.

4. TLC of acid hydrolyzed water soluble mild alkaline hydrolysis products was also done. The water soluble products from mild alkaline hydrolysis were further hydrolyzed in 1 ml of 2 N HCL at 100 C. for 3 hours to release the nitrogenous constituents. These products were run on ascending silica gel TLC plates in isopropanol:28% ammonium hydroxide (7:3) against appropriate standards.

5. Phosphatidyl glycerol was identified by detecting the free glycol group. The analysis was performed by the following procedure:

   a. Scrape the proposed phosphatidyl glycerol spot from the TLC plate into a clean test tube.

   b. Add 0.5 ml 95% ethanol to elute.

   c. Add 0.5 ml 0.5 N H_2SO_4 and 0.1 ml of freshly made 0.1 M $NaIO_4$.

   d. Incubate 15 minutes in the dark.

   e. Add 0.1 ml M $NaHSO_3$.

   f. Mix and incubate 10 minutes.

   g. Add an equal volume of chromotropic acid reagent.

   h. Heat in a boiling water bath for 30 minutes.

   i. Read optical density at 570 nanometers.

   Formaldehyde production was quantitated by referring to a standard curve prepared by treating various concentrations of formaldehyde with chromotropic acid reagent as above and plotting optical density against concentration. Phosphatidyl ethanolamine and silica gel were carried through this procedure as controls.

   Stock chromotropic acid reagent consisted of 5% aqueous chromotropic acid. Working reagent contained 1 ml stock and 10 ml concentrated H_2SO_4.

J. Argentation Chromatography

Developing a thin layer chromatogram through silica gel impregnated with silver nitrate is called argentation chromatography. Plates were prepared by spraying 3/4 of a standard silica gel TLC plate with 15% (w/v) $AgNO_3$. By leaving 1/4 of the plate free of $AgNO_3$, the first direction of development could proceed up through this strip of $AgNO_3$ free silica gel. The plate was then dried, turned 90 degrees, and developed through the $AgNO_3$ impregnated silica gel. A mixed phospholipid sample was processed in this manner developing in chloroform:methanol:water (65:25:4) in both directions.

K. Quantitation of Lipids

1. Gravimetric Analysis: Quantitation of PHB, total simple lipids, and total phospholipids by weight could be done with mg quantities of material. Samples were dried in preweighed aluminum weighing cups and weighed using an Ainsworth Analytical Balance. Whole cell lipids were compared to 20P membrane lipids.
2. Dichromate Oxidation: Lipids reduce $Cr_2O_7^{2-}$ to Cr^{3+}. Increased absorbance at 575 nanometers due to lipid oxidation was measured using a Gilford Model 2400 Recording Spectrophotometer.

Acid dichromate reagent contained 2.5 gm $K_2Cr_2O_7$ in 1 liter of concentrated H_2SO_4. Whole cell and membrane simple lipids were analyzed by the following procedure:

a. Scrape the TLC spot into a clean test tube.
b. Add 2 ml dichromate reagent.
c. Shake tubes to mix contents.
d. Incubate 45 minutes at 100 C. with periodic shaking.
e. Centrifuge to pellet silica gel.
f. Remove 1 ml of sample.
g. Dilute with 5 ml water.
h. Read optical density at 575 nanometers.

Use silica gel treated as above for the spectrophotometric blank.

3. Radioactive Phosphorus: P^{32} was used to quantitate phospholipids. To label the phospholipids, 1 mCi P^{32} as orthophosphate (Amersham-Searle) was added to 2 liters of CM medium in a vacuum flask. The flask was inoculated, gassed with methane:air (1:1), and incubated in a fume hood. During incubation, the culture was stirred with a magnetic stirring apparatus. Cells were harvested and phospholipids were obtained by the usual methods. Labelled phospholipids on a silica

gel thin layer plate were analyzed in several ways.

a. The spots were located using a Baird-Atomic Deluxe Radiochromatogram Scanner Model RSC-363. The instru- was operated at 1100 volts with a detector gas consisting of 0.95% isobutane in helium. The scanner tracing served not only to locate the spots but to quantitate them. Peak areas were measured using an A.O.Ott planimeter.

b. The phospholipid spots were scraped from the TLC plate into polyethylene scintillation vials containing 10 ml of cocktail, and radioactivity was counted using a Packard Tricarb Liquid Scintillation Spectrophotometer. The scintillation cocktail contained the following:

Cabosil (Packard)	40 gm
PPO (Packard)	5 gm
Dimethyl PPO (Packard)	0.25 gm
Toluene	1 liter

c. The TLC plate containing labelled phospholipids was also analyzed by autoradiography. A sheet of Kodak X-ray film was placed over the TLC plate, and both were stored in a light tight box for 24 hours. The X-ray film was developed in Dektol (Kodak) for 3 minutes and fixed for 10 minutes.

L. Analysis of the Fatty Acids of the Phospholipids

1. Preparation of Methyl Esters: Deacylation and methylation were accomplished by mild methanolysis (White, 1968). After elution of the phospholipids from silica gel with chloroform:methanol (3:1), the solvent was evaporated and methylation of the fatty acids was achieved as follows:

   a. Dissolve the lipid in 1 ml methanol:toluene (1:1).

   b. Add an equal volume of 0.2 M KOH in methanol.

   c. Incubate at 0 C. for 2 hours.

   d. Neutralize with ion exchange resin (BioRex-70, Bio Rad).

   e. Add 1 volume of chloroform, 1 volume of water, and mix thoroughly.

   f. After the phases separate, recover the methyl esters as the lower hydrophobic layer.

   The sample was kept under N_2 where applicable to prevent oxidation of labile fatty acids.

2. Gas Chromatography of the Methyl Esters: Separation of of the fatty acid methyl esters was achieved using a Varian Aerograph Model 200 Gas Chromatograph equipped with a flame ionization detector and a 8 ft X 1/8 inch diethylene glycol succinate column. Injector and detector were 200 C. while the column was 170 C. Nitrogen carrier

gas was adjusted to 25 ml/min. Various size samples were injected with a 100 microliter syringe (Hamilton).

3. Detection of Unsaturation: Since saturated and mono-unsaturated fatty acids come off the column very close to one another, a large peak in the vicinity could be either one. The sample was mixed with a few drops of bromine water, and the resulting chromatogram was compared with that of an untreated sample. Bromine will change the column retention time of an unsaturated fatty acid.

VI. Membrane Protein Characterization

A. Amino Acid Composition

Amino acid composition was determined by a thin layer chromatographic system that resulted in separation of polar amino acids (i.e. glutamic acid, aspartic acid, lysine and arginine) from the apolar amino acids (i.e. all other amino acids). Membrane preparations were analyzed as follows:

1. Hydrolyze the sample in 6 N HCl for 36 hours at 110 C.
2. Evaporate the HCl, add water, and evaporate again.
3. Dissolve the amino acids in 0.1 ml water.
4. Spot the amino acid mixture on a silica gel TLC plate.
5. Develop in 95% ethanol:28% ammonium hydroxide (7:3).

6. Scrape the polar and apolar amino acid fractions, determined by running appropriate standards, into test tubes.
7. Elute the amino acids with 2 ml water.
8. Pellet the silica gel by centrifugation.
9. Remove 1 ml of sample, and quantitate the amino acids by the procedure described in the following section.

B. Amino Acid Quantitation

Amino acids were quantitated by the procedure of Rosen (1957). The following reagents were required for this analysis.

1. 0.01 M NaCN
2. sodium acetate 2.7 kg
   glacial acetic acid 500 ml
   distilled water 2 liters
   pH 5.3-5.4
3. Buffer: 2 ml #1 and 98 ml #2
4. 3% (w/v) ninhydrin in methyl cellosolve
5. isopropanol:water (1:1) diluent

The procedure used was as follows:

1. To 1 ml sample, add 1/2 ml #3 and 1/2 ml #4.
2. Heat 15 minutes in boiling water.
3. Add 5 ml diluent and shake vigorously.

5. Read optical density at 570 nanometers.

Amino acid concentration was read from a standard curve prepared by analyzing various concentrations of alanine and plotting optical density versus concentration.

C. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was used to examine the size of membrane proteins. Proteins were solubilized from membranes by heating for 5 minutes in 5% sodium dodecyl sulfate (SDS) at 100 C. The sample was mixed with 1/2 volume of glycerol to facilitate layering on the gel.

The electrophoretic buffer was 0.05 M phosphate containing 0.1% SDS (w/v). The gel contained 7.5% acrylamide (Eastman Organic Chemicals) and 0.1% (w/v) methylene-bis-acrylamide (Eastman Organic Chemicals) in the SDS buffer. The mixture was polymerized with 0.2 ml of freshly made ammonium persulfate (Bio Rad) and 0.02 ml N,N',N',-tetramethylethylenediamine (Bio Rad).

Using a multitube electrophoresis apparatus, the gels were washed at 5 amps/gel for 30 minutes. Samples were then layered on the gels with a pipette, and 2 amps/gel was applied for 30 minutes to move the samples into the gels. The gels were then run at 5 amps/gel for 1 hour. The gels were stained with

0.1% Amido Black (w/v) in ethanol:glacial acetic acid:water (20:7:73). The gels were decolorized and stored in 7% (v/v) glacial acetic acid. Bovine serum albumin (Sigma) was used as a standard. It contains a monomer with a molecular weight of 60,000 and a dimer with a molecular weight of 120,000. Molecular weights of membrane proteins were read from a standard curve prepared by plotting the log of the molecular weight of the BSA standard versus migration distance.

D. Cytochrome Characterization

Membrane cytochromes were examined using a Shimadzu Model MPS-50L Recording Spectrophotometer. Samples were scanned from 350 to 650 nanometers against a buffer blank or alternatively, oxidized versus reduced samples were used. Samples were reduced by adding a few grains of sodium dithionite and oxidized by adding a drop of hydrogen peroxide.

Various substrates were tested for their ability to reduce cytochromes in a 10S fraction. Methane was added as a drop of methane saturated buffer or by bubbling gas through the cuvette. Methanol was added by the drop. Formaldehyde and formic acid were added dropwise as aqueous solutions.

VII. Enzyme Assays

Cells were grown, disrupted, and centrifuged differentially as explained previously except the 20S fraction was centrifuged for 30 minutes at 100,000 X g. The 100,000 g pellet (100P) suspended in 0.05 M phosphate buffer pH 7 was used as a membrane enzyme preparation and the 100S fraction was used as a extra-membrane enzyme preparation. The sources of the chemicals used in the enzyme assays will be listed at the end of this section.

A. L-serine:tetrahydrofolate 5,10-hydroxymethyl transferase

This enzyme was assayed spectrophotometrically by measuring the reduction of NADP as described by Hatefi et al. (1957). The reaction mixture contained the following:

serine	10 micromoles
tetrahydrofolic acid	0.6 micromoles
NADP	0.6 micromoles
sodium phosphate (ph 7.5)	100 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

Change in optical density at 340 nanometers was measured using a Gilford Model 2400 Recording Spectrophotometer. The extinction coefficient of NADH and NADPH is 6.22 X 10^6 cm^2/mole. Controls containing NADPH, everything but substrate, and everything but enzyme were also run.

B. Formate:NAD Oxidoreductase

This enzyme was assayed as explained in the preceding section except NAD reduction was measured. The reaction mixture contained the following:

sodium phosphate (pH 7.5)	100 micromoles
NAD	10 micromoles
sodium formate	20 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

C. Formaldehyde:NAD Oxidoreductase

This enzyme was assayed as in part B. The reaction mixture contained the following:

sodium phosphate (pH 7.5)	100 micromoles
NAD	10 micromoles
Formaldehyde	20 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

D. D-glycerate:NAD Oxidoreductase

This enzyme was assayed spectrophotometrically as explained previously except NADH oxidation was measured. The reaction mixture contained the following:

sodium phosphate (pH 7.5)	100 micromoles
NADH	0.5 micromoles
3-hydroxypyruvate	10 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

E. 3-hydroxybutyrate:NAD Oxidoreductase

This enzyme was assayed spectrophotometrically as in section B. The reaction mixture contained the following:

sodium phosphate (pH 7.5)	100 micromoles
NAD	10 micromoles
3-hydroxybutyrate	20 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

F. Methanol:NAD Oxidoreductase

This enzyme was assayed spectrophotometrically as explained in part B. The reaction mixture contained the following:

Tris (pH 9)	0.3 millimoles
Methanol	20 micromoles
NH_4Cl	45 micromoles
NAD	10 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

G. NAD Independent Methanol Dehydrogenase

This enzyme was assayed similar to the preceding assay except NAD was deleted from the above reaction mixture and 3.3 micromoles phenazine methosulfate and 0.13 micromoles dichlorophenolindophenol were added. Optical density was measured at 600 nanometers. The Molar extinction coefficient of DCIP at 600 nanometers is 1×10^6.

H. NADH:Cytochrome C Oxidoreductase

This enzyme was assayed spectrophotometrically by measuring cytochrome reduction at 550 nanometers. The reaction mixture contained the following:

sodium phosphate (pH 7.5)	100 micromoles
cytochrome C (oxidized)	0.5 micromoles
NADH	0.5 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

I. ATP Phosphohydrolase

This enzyme was assayed by measuring the release of inorganic phosphate from ATP after 5 minutes reaction time. Controls minus substrate or enzyme were also analyzed. The reaction mixture contained the following:

ATP	100 micromoles
Histidine Buffer (pH 7.5)	200 micromoles
$MgCl_2$	5 micromoles
enzyme preparation	0.1 ml
water	to make 3 ml

The histidine buffer was prepared as 0.2 M histidine in 0.15 M KCl, pH 7.5. The inorganic phosphate assay was

a mofification of that of Ames (1966). The following reagents were required for the assay:

1. ascorbic acid 10% (w/v)
2. ammonium molybdate 0.42% (w/v) in 1 N H_2SO_4
3. working reagent: 1 part #1 and 6 parts #2

The test was performed by adding 0.7 ml working reagent to 0.3 ml sample and incubating 20 minutes at 45 C. Optical density was read at 800 nanometers. Inorganic phosphate concentration was read from a standard curve prepared by analyzing various concentrations of sodium phosphate using this procedure and plotting phosphate concentration versus optical density.

Histidine, cytochrome C, NADH, 3-hydroxybutyrate, 3-hydroxypyruvate, serine, tetrahydrofolic acid, NADP, and phenazine methosulfate were obtained from Nutritional Biochemicals Corp. ATP and NAD were obtained from General Biochemicals Corp. Tris was obtained as Trizma Base from Sigma Chem. Corp. Methanol, ammonium chloride, formaldehyde, and ammonium molybdate were obtained from J.T.Baker Co. DCIP and sodium formate were purchased from Fisher Chem. Co. Ascorbic acid was obtained from Merck and Co.

RESULTS AND DISCUSSION

I. Growth of the Bacteria

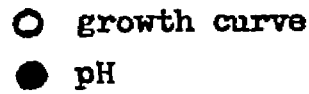
Methylomonas methanica formed round pink colonies on CM agar and a red-pink pellicle in liquid CM medium. The bacteria were 0.7 X 1.5 micrometer rods. This bacterium formed a very thick (i.e. 1/2 inch) pellicle in enrichment cultures, but formed only a thin film on the surface of the medium in pure culture. By microscopic examination, the pellicle formed by the enrichment cultures appeared to consist almost entirely of Methylomonas methanica; however, other bacteria in the pellicle were obvious when the culture was plated on nutrient agar. Thus, the thick pellicle did not seem to result from proliferation of other bacteria in the enrichment, rather, relatively few of these bacteria seemed to stimulate M. methanica to produce the thick pellicle.

Relatively light growth of Methylosinus trichosporium was also observed on both liquid and solid media. For example, it took about 3 weeks for colonies to reach 1 millimeter in diameter on CM agar plates; in liquid culture, yields of 0.2 gm/liter (wet weight) were observed. If the growth of M. trichosporium in mixed culture is analogous to Methylomonas methanica, the rates and quantities of methane oxidation may even be greater in the natural environment where mixed populations of bacteria are the maxim. This raises

some question as to the relevance of pure culture studies and conclusions drawn from such studies as they relate to growth in the natural habitat.However, this cannot prevent continuence of pure culture studies since they are essential for controlled experiments; nevertheless, one should keep these shortcomings in mind.

The growth response of M. trichosporium in CM medium is illustrated in Figure 1. The curve resembles a typical bacterial growth curve. The gradual decline in pH in the medium is probably due to carbon dioxide production since formic acid did not accumulate. However, since a complete analysis of the post-growth medium was not done, the possibility of some other acidic product can not be ruled out. As is evident in the growth curve, bacterial numbers declined after the fifth day. For this reason, culture transfers were made weekly. If culture transfer was delayed longer than 2 weeks, there was an excessive lag period prior to the appearance of visible growth. This was apparently due to cell die off after the first week as suggested by the growth curve. Estimations of growth parameters can be obtained from these data. The logarithmic growth phase is restricted to the time period prior to 24 hours growth and probably even to less than 12 hours. Since

Figure 1. Growth curve for Methylosinus trichosporium cultivated in liquid CM medium at 21 C. and pH 7.



Bacteria / ML.
10^7
10^8
10^9
0
1
2
3
4
5
6
7
Time (days)
6.0
6.5
7.0
PH

there may be a lag phase in this growth response, rates calculated from these data may be slightly lower than actually exists.

Logarithmic phase bacteria growth can be represented by the following function:

$$N = N_0 \, e^{ct}$$

N represents the number of bacteria at time t, N_0 is the number of bacteria at time zero, and c is a growth constant. By using the numbers of bacteria at time 0 and 12 hours in the growth curve as an approximation of logarithmic growth, the growth constant was calculated to be 0.1312 $hours^{-1}$. By knowing the growth constant, bacterial numbers at any time during logarithmic growth can be predicted, and bacterial generation time can be calculated. By definition, $N = 2N_0$ when t = generation time (GT). By substituting $2N_0$ for N in the general equation and taking the natural log of both sides, the following equation was obtained:

$$\ln 2 = (0.1312 \text{ hours}^{-1})(GT)$$

Solving this equation for GT, the generation time was found to be 5.3 hours. This is somewhat longer than the 3 hour generation

time reported by Vary and Johnson (1967) for a mixed culture of methane oxidizing bacteria and may simply reflect the pure versus mixed culture growth. Alternatively, M. trichosporium may have an intrinsically longer generation time than the bacteria of Vary and Johnson. The value corresponds closely with the report of Whittenbury (1969) of generation times around 5 hours for Methylosinus isolates.

Whittenbury (1970) examined M. trichosporium for its ability to utilize 7 amino acids, 11 carbohydrates, and 7 organic acids as sole carbon and energy sources. He found that the organism was unable to grow on any substrates tested except methane or methanol. The results of a more extensive nutritional screening study involving 19 carbohydrates, 19 amino acids, 4 alcohols, and 4 organic acids were all negative; no growth was observed under aerobic or anaerobic conditions with any of the compounds listed in Table 2 nor was growth observed on methanol. This latter observation probably reflects a strain difference between the M. trichosporium used in this study and that tested by Whittenbury. Therefore, this organism appears to be an obligate methylotroph incapable of growth on any substrate other than methane. This does not imply inability of this organism to metabolize other organic compounds.

Figure 2 illustrates the effects of various organic acids on methane oxidation by M. trichosporium; Figure 3 shows effects of

Table 2. Organic compounds tested for the ability to support growth of Methylosinus trichosporium.

Carbohydrates	Amino Acids	Organic Acids
adonitol	alanine	acetic acid
arabinose	asparagine	butyric acid
dulcitol	aspartic acid	formic acid
galactose	cysteine	propionic acid
glucose	glutamic acid	
inositol	glycine	Alcohols
inulin	histidine	butanol
levulose	hydroxproline	ethanol
maltose	isoleucine	methanol
mannitol	lysine	propanol
mannose	methionine	
melibiose	ornithine	
raffinose	phenylalanine	
rhamnose	proline	
salicin	serine	
sorbitol	threonine	
sorbose	tryptophane	
trehalose	tyrosine	
xylose	valine	

various amino acids; Figure 4 represents the effects of two pentoses; Figure 5 shows effects of several hexoses. These data show a general enhancement of methane oxidation by the organic compounds. This enhancement appeared as an increased initial rate of methane oxidation and a greater total amount of methane oxidized. All the graphs have general similarities. The bottles containing only cells oxidized methane at a relatively steady rate while the bottles to which organic compounds were added showed more rapid initial methane oxidation leveling off to some lesser rate. The organic acids elicited the greatest stimulation which was about a 4 fold increase in methane oxidation on the first day. The amino acids and pentoses resulted in a 3 fold increase in methane oxidation on the first day, while the hexoses enhanced methane oxidation only slightly. The relatively slight stimulation by the hexoses may be due to a permeability effect.

The explanation for these observations is presently unknown. Perhaps the organic compounds stimulated cell division thereby increasing the number of methane oxidizing bacteria and the quantity of methane oxidized. Alternatively, the organic compounds could have affected the rate of methane oxidation without increasing cell numbers. This could have been done by directly affecting the methane oxidation process or by increasing the solubility and/or availability of methane to the bacteria. Regardless of the

Figure 2. Curves showing micromoles of methane oxidized by *Methylosimus trichosporium*. Curves represent 10^{-2} M concentrations of various organic acids.

- ● Control-sterile CM medium
- ○ Medium + cells
- ■ Medium + cells + sodium formate
- △ Medium + cells + sodium acetate
- □ Medium + cells + sodium propionate
- ▽ Medium + cells + sodium butyrate

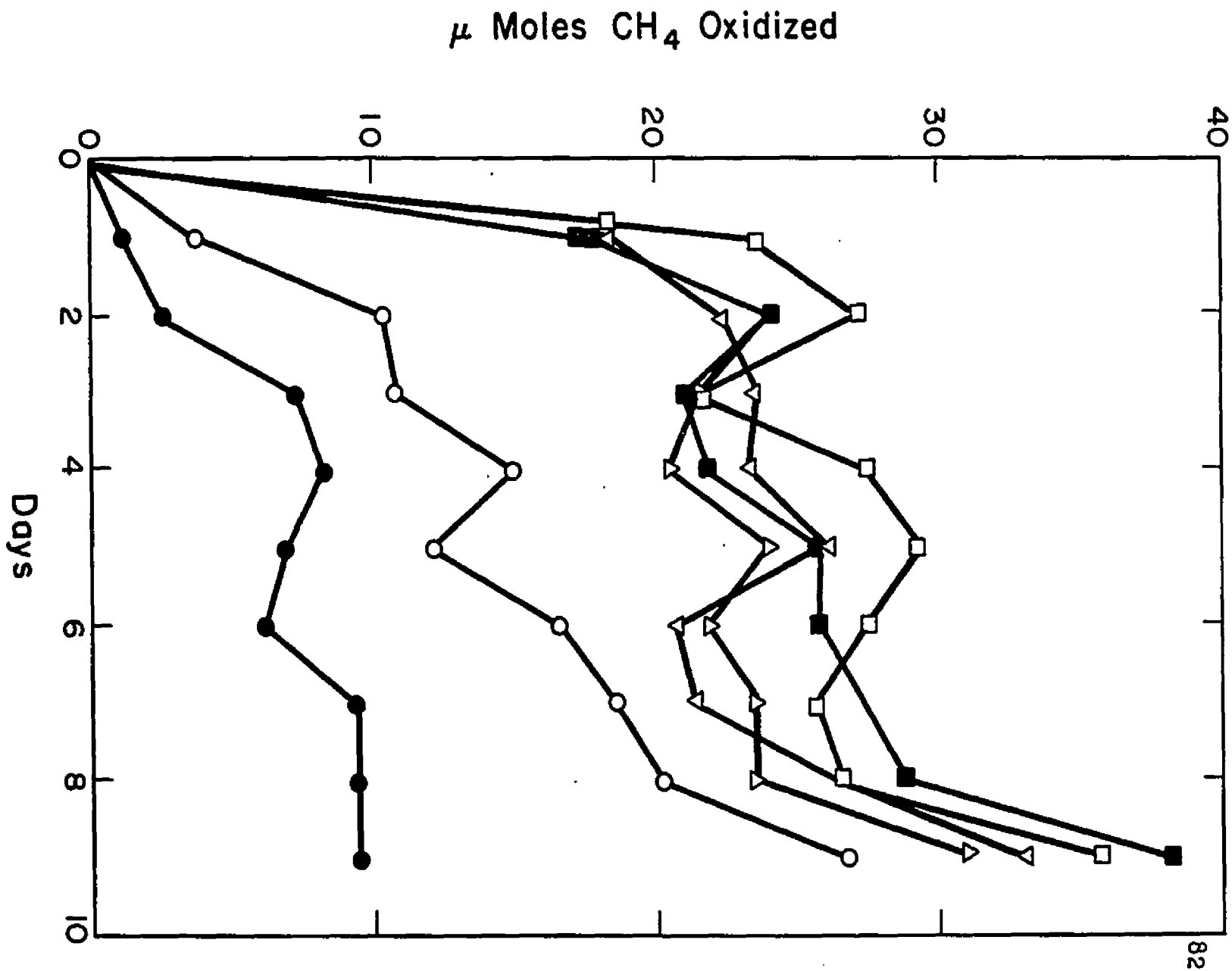

μ Moles CH_4 Oxidized
0
10
20
30
40
Days
0
2
4
6
8
10
82

Figure 3. Curves showing micromoles of methane oxidized by *Methylosinus trichosporium*. Curves represent 10^{-2} M concentrations of various amino acids.

- ● Control-sterile CM medium
- ○ Medium + cells
- △ Medium + cells + serine
- □ Medium + cells + alanine
- ▣ Medium + cells + glutamic acid

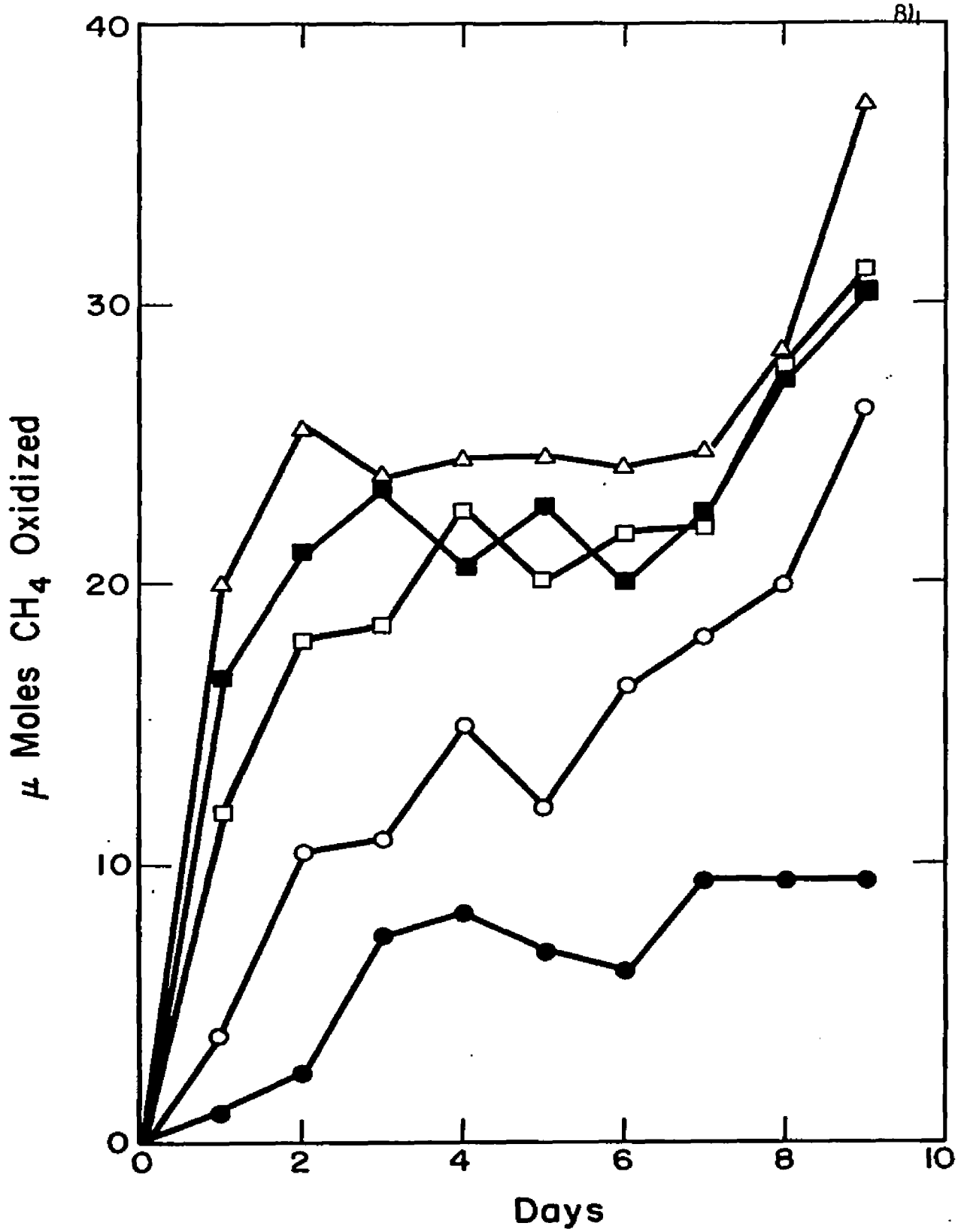

40
30
20
10
0
0
2
4
6
8
10
μ Moles CH_4 Oxidized
Days

Figure 4. Curves showing micromoles of methane oxidized by *Methylosinus trichosporium*. Curves represent 10^{-2} M concentrations of various pentoses.

- ● Control-sterile CM medium
- ○ Medium + cells
- □ Medium + cells + arabinose
- ■ Medium + cells + xylose

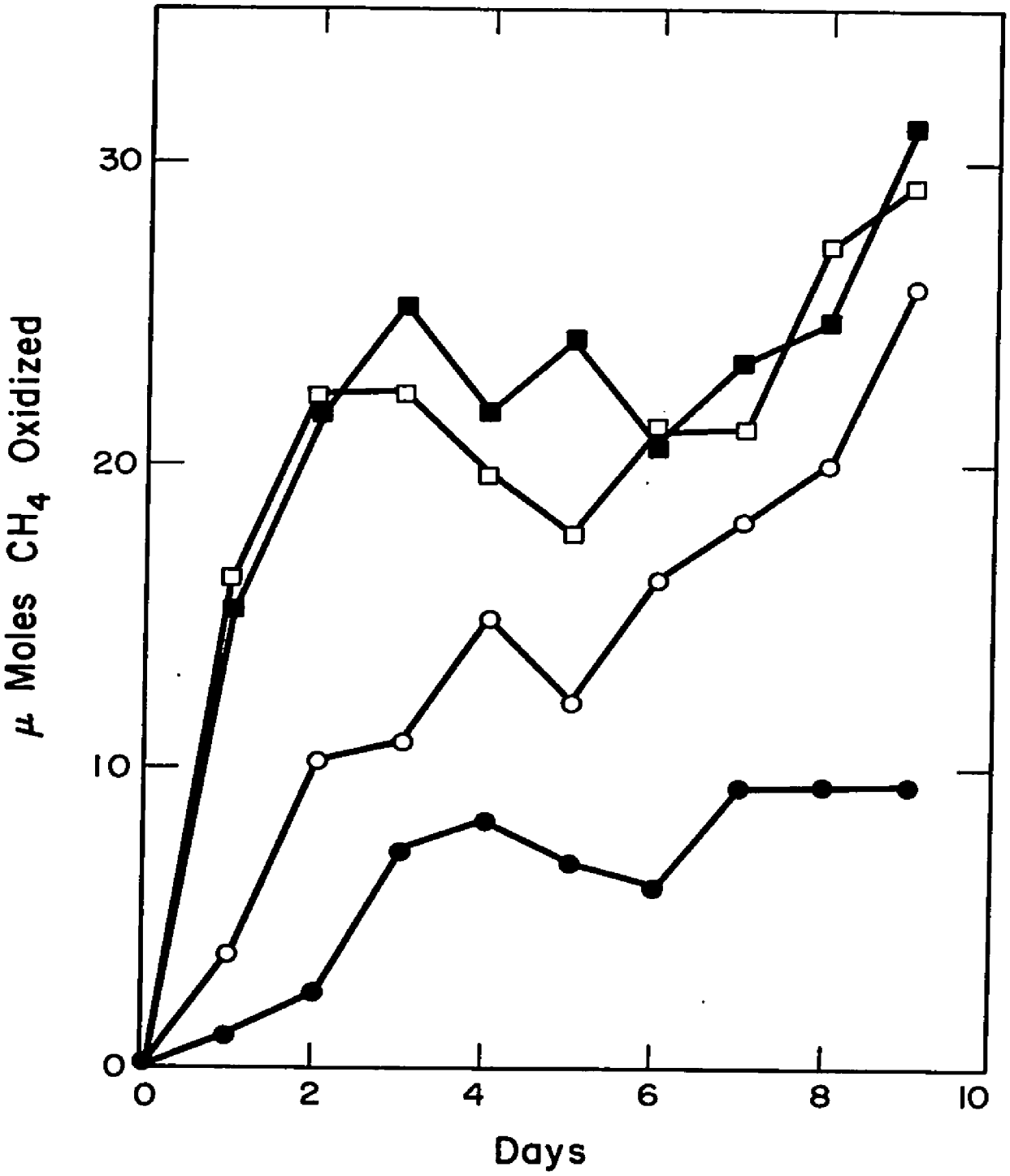
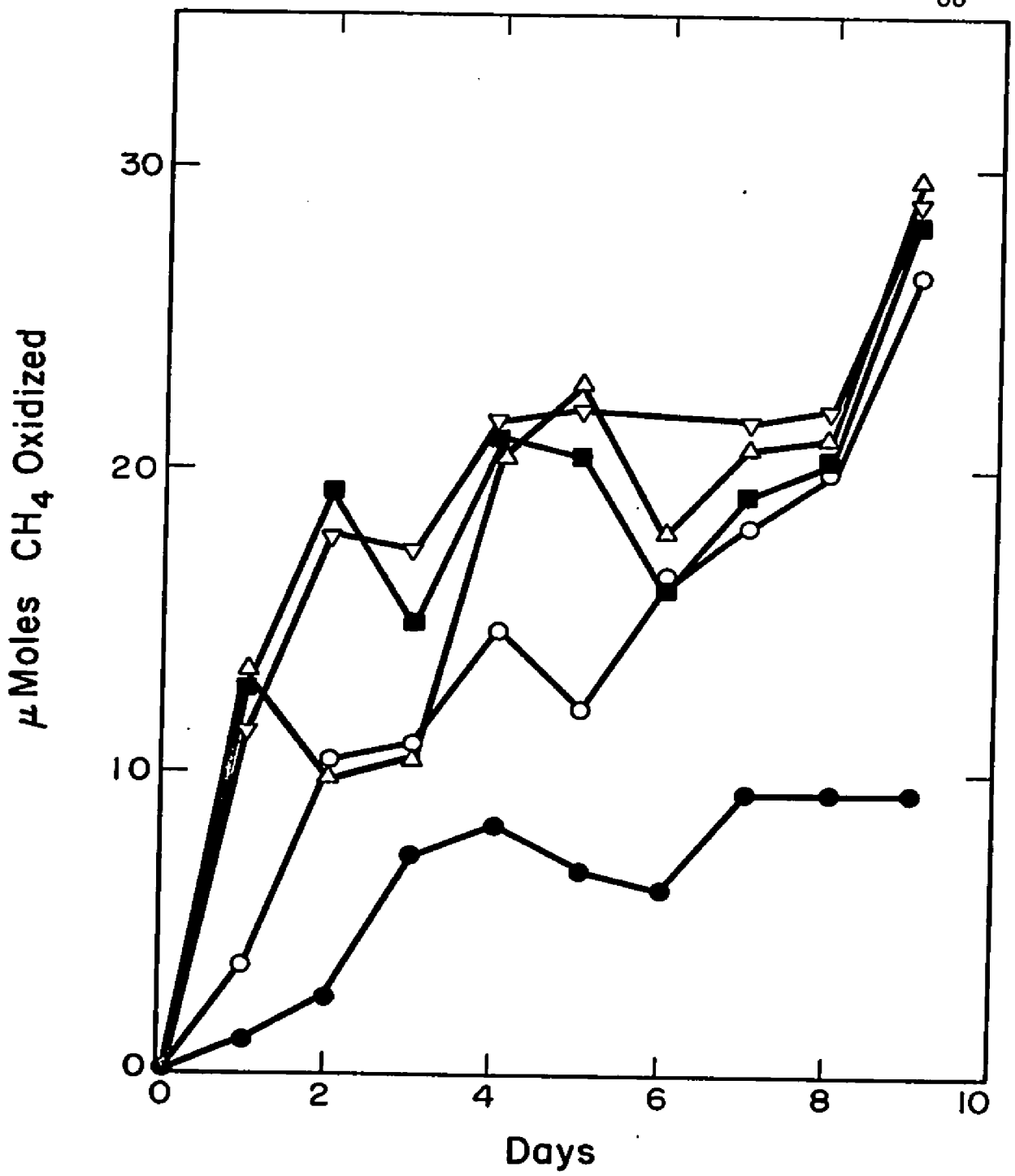

μ Moles CH_4 Oxidized
0
10
20
30
0
2
4
6
8
10
Days

Figure 5. Curves showing micromoles of methane oxidized by _Methylosinus trichosporium_. Curves represent 10^{-2} M concentrations of various hexoses.

- ● Control-sterile CM medium
- ○ Medium + cells
- ■ Medium + cells + glucose
- △ Medium + cells + galactose
- ▽ Medium + cells + mannose


30
20
10
0
0
2
4
6
8
10
Days
μMoles CH4 Oxidized

explanation, this phenomenon would have a tremendous effect on these bacteria in the natural environment. By secretion, leaking, or lysis, organisms of all kinds release variable quantities and types of organic materials into the environment that would affect methane oxidation by these bacteria. Less widespread but significant in restricted situations would be similar stimulating effects due to external organic pollutants entering aquatic ecosystems.

Results of the determination of gaseous substrate and product balances during methane oxidation by M. trichosporium are shown in Table 3. By comparing the amount of methane oxidized with the amount of carbon dioxide produced, it appears that about 50% of the methane that was oxidized was incorporated into cellular material. Therefore, only half as much carbon dioxide was produced as was methane oxidized. Since complete oxidation of methane to carbon dioxide via methanol, formaldehyde, and formate involves 4 oxidations, and because methane is incorporated at the formaldehyde level, the incorporation of 50% of the methane oxidized into cellular material decreases the number of oxidations from 8 per 2 methane molecules to 6 per 2 methane molecules. Therefore, the potential energy yielding oxidations were decreased by 25% while 50% of the methane was fixed into cell material.

Another interesting feature is the relationship between methane oxidized to oxygen consumed which was about 6 atoms of

Table 3. Gaseous substrate and product balances during methane oxidation by Methylosinus trichosporium grown in CM medium at 21 C.

Time (days)	UTILIZATION (micromoles/ml)		PRODUCTION (micromoles/ml)		RATIOS	
	CH_4	O_2	N_2	CO_2	O_2/CH_4	CH_4/CO_2
1	1.67	2.85	0	0.74	1.7	2.2
2	4.69	7.06	0	2.27	1.5	2.1
3	4.75	7.64	0	2.39	1.6	2.0

oxygen per 2 molecules of methane oxidized. Based upon incorporation of 50% of the methane at the formaldehyde level, there was 1 oxygen utilized per oxidative step. This stoichiometry is consistent with either a mixed function oxidase or a hydration-dehydrogenation for the initial oxidation of methane. In view of the studies of Leadbetter and Foster (1959) and Higgens and Quayle (1970) showing O^{18} incorporation into methane oxidizing bacteria, M. trichosporium probably also utilizes a mixed function oxidase (monooxygenase) at the first oxidation. The relationship between methane and oxygen consumed shown in Table 3 suggests a 1:1 ratio for the initial oxidation or the following reaction:

$$2\ CH_4 + O_2 \rightarrow 2\ CH_3OH$$

This is as opposed to a mechanism involving 2 oxygen atoms per methane in the initial oxidation with some reduced hydrogen donor transporting hydrogen to the extra oxygen. The oxidation of methane to methanol has a $G_0' = -26.12$ Kcal $mole^{-1}$ (Ribbons et al., 1970). Although it is energetically feasible to recover ATP from this reaction, coupling of phosphorylation to monooxygenase has yet to be demonstrated. The remaining oxidative steps in the oxidation of methane are probably dehydrogenations coupled with electron transport and oxidative phosphorylation. Further evidence to support these conclusions will be discussed later with the cytochrome studies.

II. Morphology of the Cells and Their Intracytoplasmic Membranes

In Figure 6, M. trichosporium is observed to be a smoothly textured rod shaped bacterium. Measurements from a variety of electron micrographs revealed that the bacterium is 2-3 micrometers long and may be somewhat pear-shaped ranging from 0.5-1 micrometer in width. Figure 6 also shows the cell wall to be 15.4 nanometers thick with a distinct outer region 6 nanometers thick and an inner region 9.4 nanometers thick. This is similar to the cell wall of Escherichia coli which has an outer layer about 5 nanometers thick and an inner layer about 8.5 nanometers thick (Davis et al., 1968). These dimensions are smaller than Gram positive cell walls which are usually between 200 and 800 nanometers thick. The outer wall of the organism was seen to be rather smooth. This is similar in appearance to the somewhat smaller M. methanica shown for comparison in Figure 7.

Figure 8 shows slightly more extensive fracturing of M. trichosporium than was observed in the first freeze etching. Again the 2 layered cell wall is evident. Also, small fibers 10-70 nanometers long can be seen connecting the cell wall and plasma membrane. The fibers attach the plasma membrane to the cell wall which made it extremely difficult to obtain purified plasma membranes from these cells. These fibers have plagued membrane investigators working with Gram negative bacteria. Of further

Figure 6. Electron micrograph of a freeze etch preparation of M. trichosporium showing:

OCW = outer cell wall
ICW = inner cell wall
S = direction of shadow

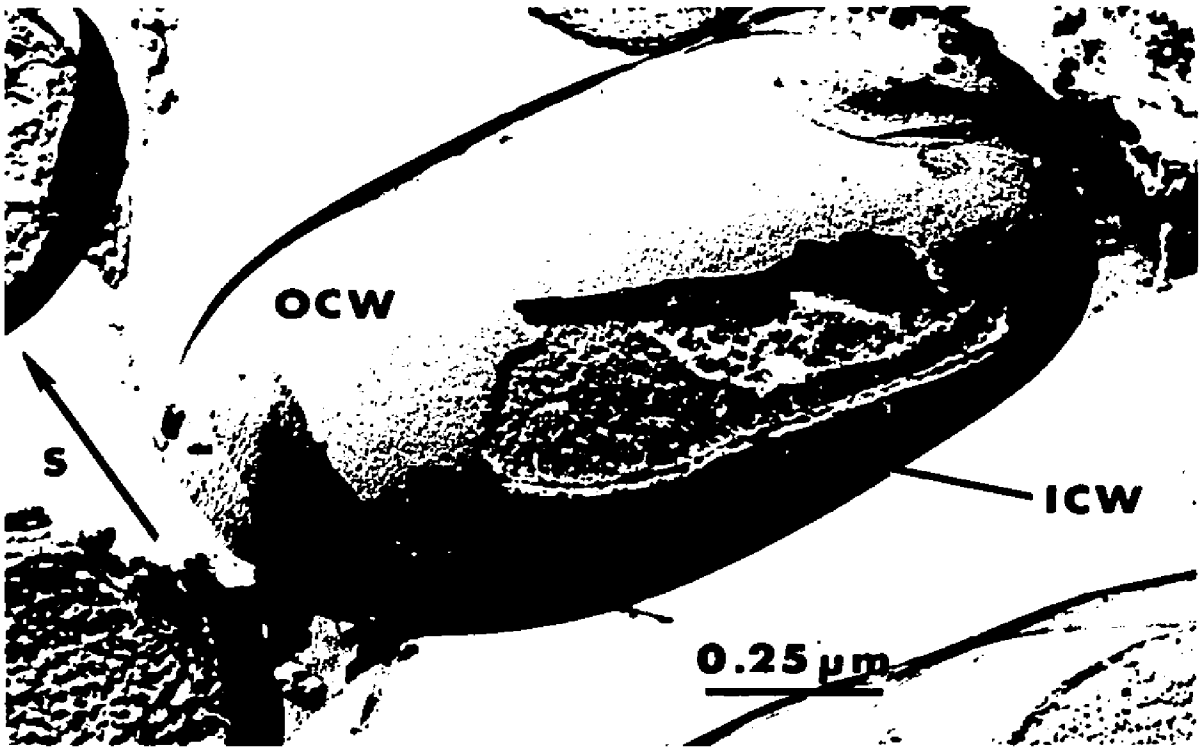

OCW
S
ICW
0.25 µm

Figure 7. Electron micrograph of a carbon replica of M. methanica. The arrow denotes the direction of shadowing.

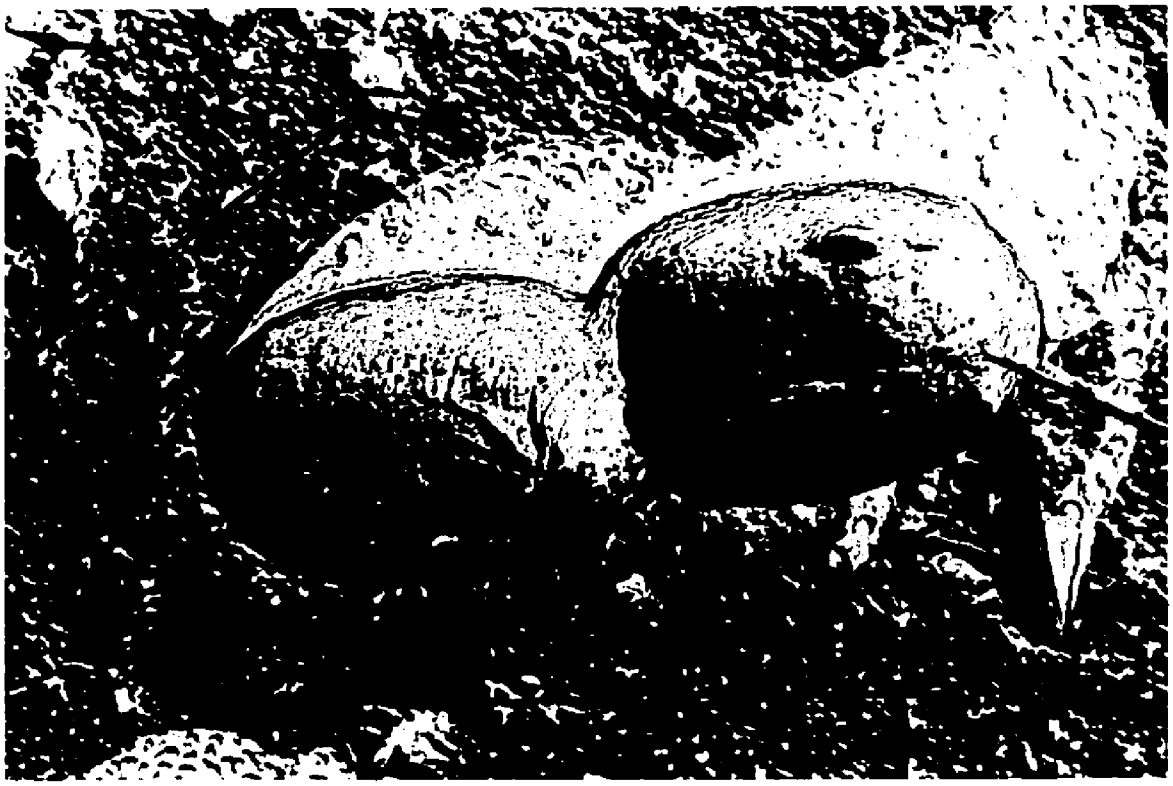


Figure 8. Electron micrograph of a freeze etch preparation of M. trichosporium showing:

F = fibers
PM = plasma membrane
V = vesicles
S = direction of shadowing


S
F
PM
V

significance in Figure 8 are balloonlike intracytoplasmic membrane vesicles (V) where the cleavage plane broke over the surface of the vesicles rather than through them. This cleavage plane fracturing which resulted in the relief evident in this electron micrograph is one of the main advantages of freeze etching as compared to thin sections. Furthermore, this is in contradiction to Whittenbury's (1969) description of the intracytoplasmic membranes as tubules.

Figure 9 shows a longitudinal fracture of M. trichosporium midway through the cell that is very similar to what one obtains with thin sections. The internal membranes are arranged in groups of vesicles 16-300 nanometers thick sometimes running the entire width and length of the cell near the periphery. Figure 10 is a transverse fracture showing essentially the same vesicular structure as in Figure 9. This reveals that the internal membranes may occupy almost all the peripheral cell area in these bacteria. In Figure 11, and enlargement of Figure 10 shows that these vesicle membranes are rather typical looking 9 nanometer thick membranes. The three dimensional effect in Figure 12 further emphasizes the extent of the internal membrane system.

The origin of these intracytoplasmic membranes is uncertain. In none of the electron micrographs could the plasma membrane be

Figure 9. Electron micrograph of a freeze etch preparation of *M. trichosporium* showing:

SV = stacked vesicles
V = vesicle
DS = direction of shadowing

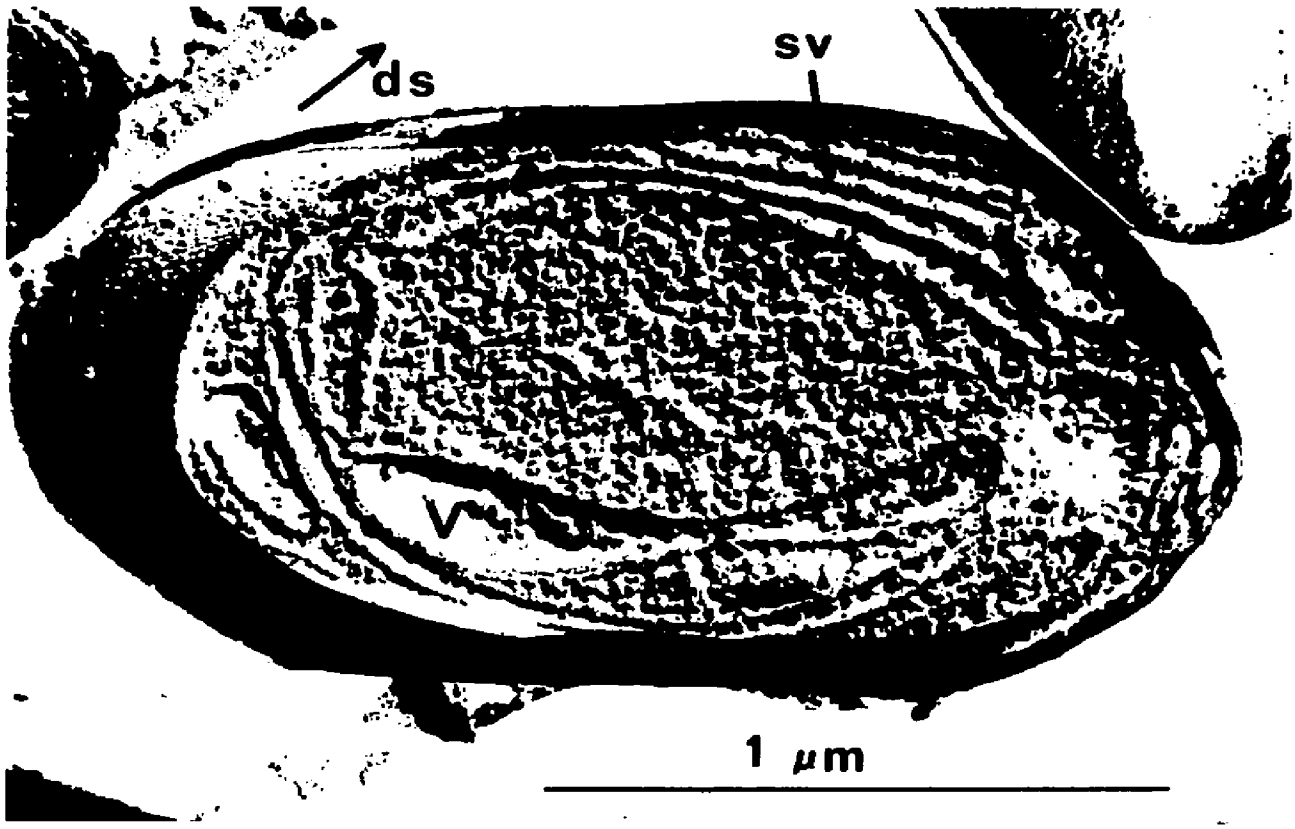

ds
sv
V
1 μm

Figure 10. Electron micrograph of a freeze etch preparation of *M. trichosporium* showing:

V = vesicle
S = direction of shadowing


V
S
0.5 µm

Figure 11. Electron micrograph of a freeze etch preparation of *M. trichosporium* showing:

V = vesicle
S = direction of shadowing

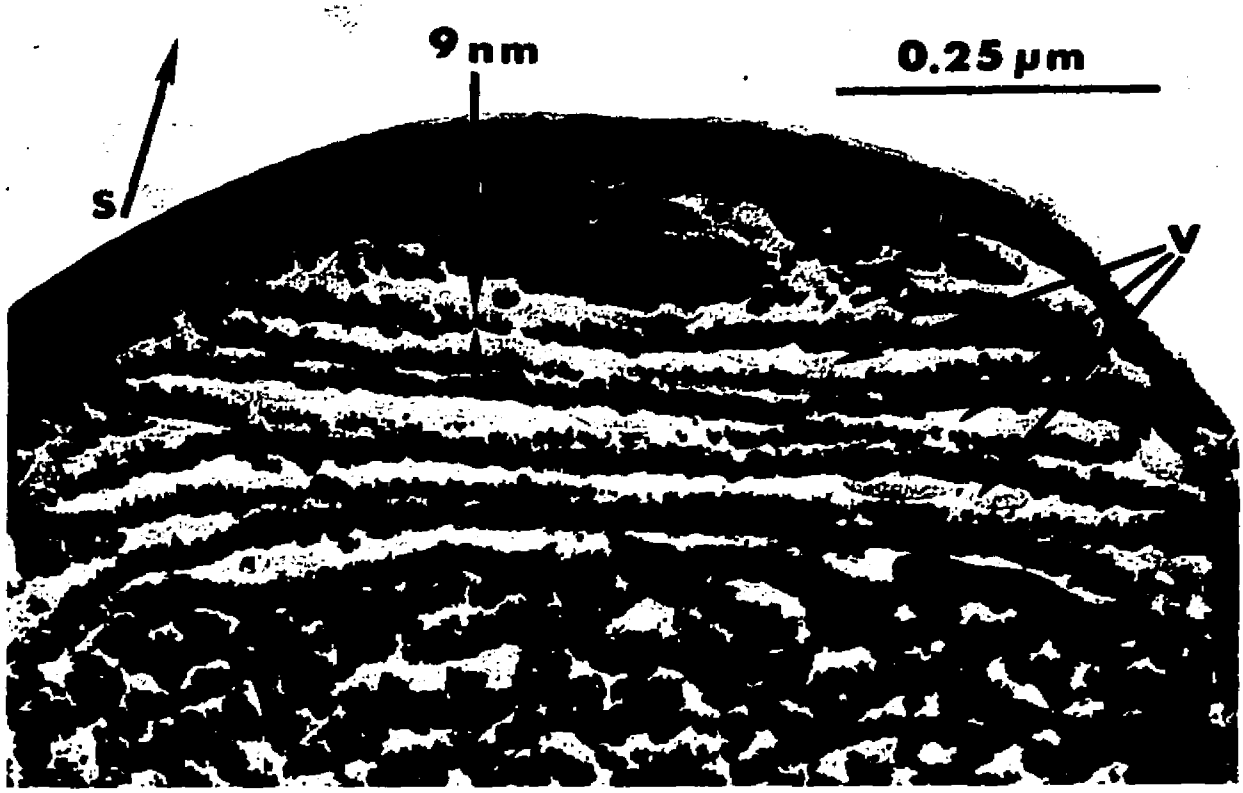

9 nm
0.25 µm
S
V

Figure 12. Electron micrograph of a freeze etch preparation of M. trichosporium showing extent of internal membranes.

S = direction of shadowing


S

seen to be continuous with or in the process of forming vesicles, however, this does not eliminate the possibility that the internal membranes originate in the plasma membrane. In at least some cases though, the internal membranes are passed from mother cell to daughter as shown in Figure 13. This electron micrograph shows a cell in the process of budding. These organisms may divide by binary fission or by budding, the latter preceding the formation of a spore. A fracture through a spore is shown in Figure 14. The complex system of outer layers, membranes, etc. suggests that the membranes passed along during budding may be important in the formation of the spore.

Figure 15 not only shows the peripheral membrane vesicles but also reveals a rodlike structure 4.5 nanometers in diameter with a repeating subunit every 4.5 nanometers. Although different in appearance from the membranes, the size of this structure suggests that it may be membrane material (i.e. 1/2 a membrane). The structure could also be a virus. Figure 16 shows another of these rodlike structures and also shows intercellular bridges (B) 30 X 75 nanometers in size that are of unknown origin and unknown function. In Figure 17, the bacteria from a liquid culture can be seen in short chains due to these intercellular connections.

Figure 18 reveals granules of PHB that range in size from 0.2-0.25 micrometers in diameter. The PHB granules were observed near the centers of cells, neither within the membrane vesicles nor

Figure 13. Electron micrograph of a freeze etch preparation of budding M. trichosporium cell showing the division of membranes.

S = direction of shadowing

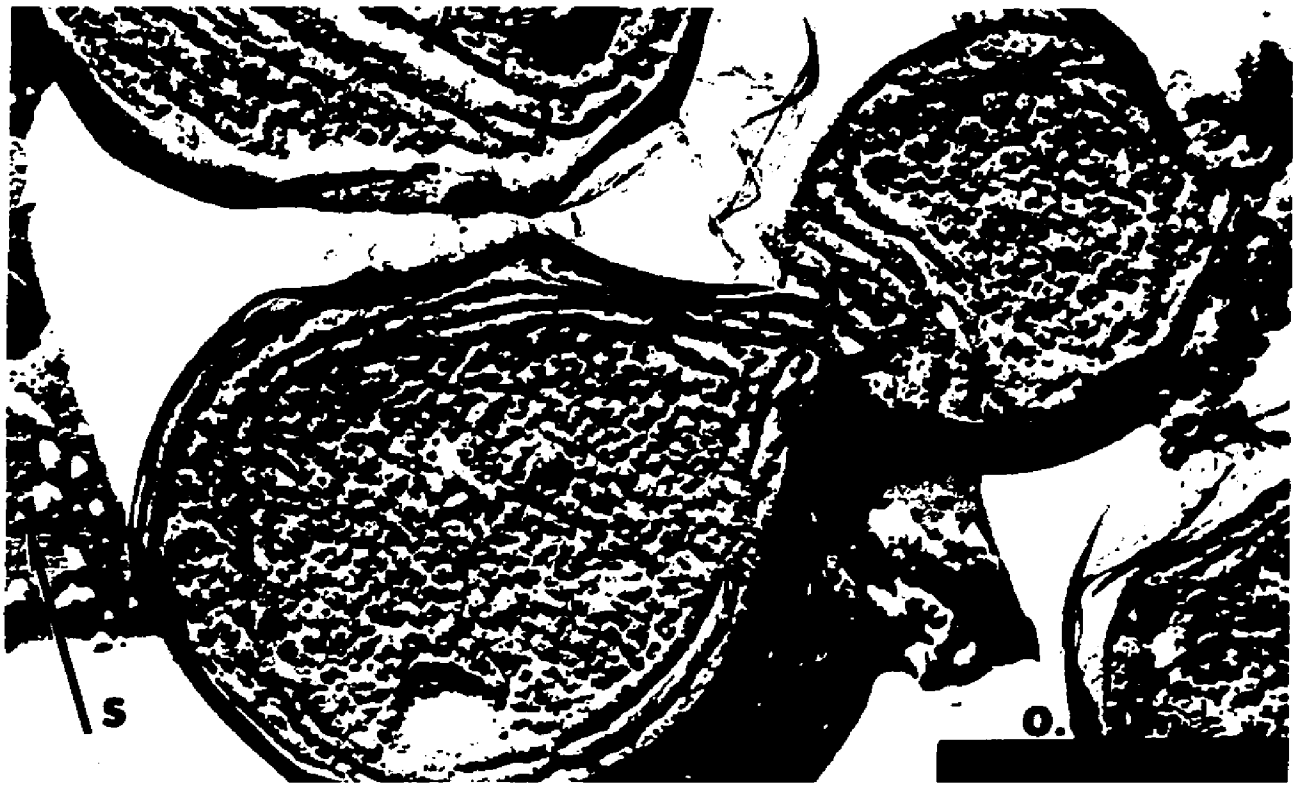

S
O.

Figure 14. Electron micrograph of a freeze etch preparation of a M. trichosporium spore.

S = direction of shadowing

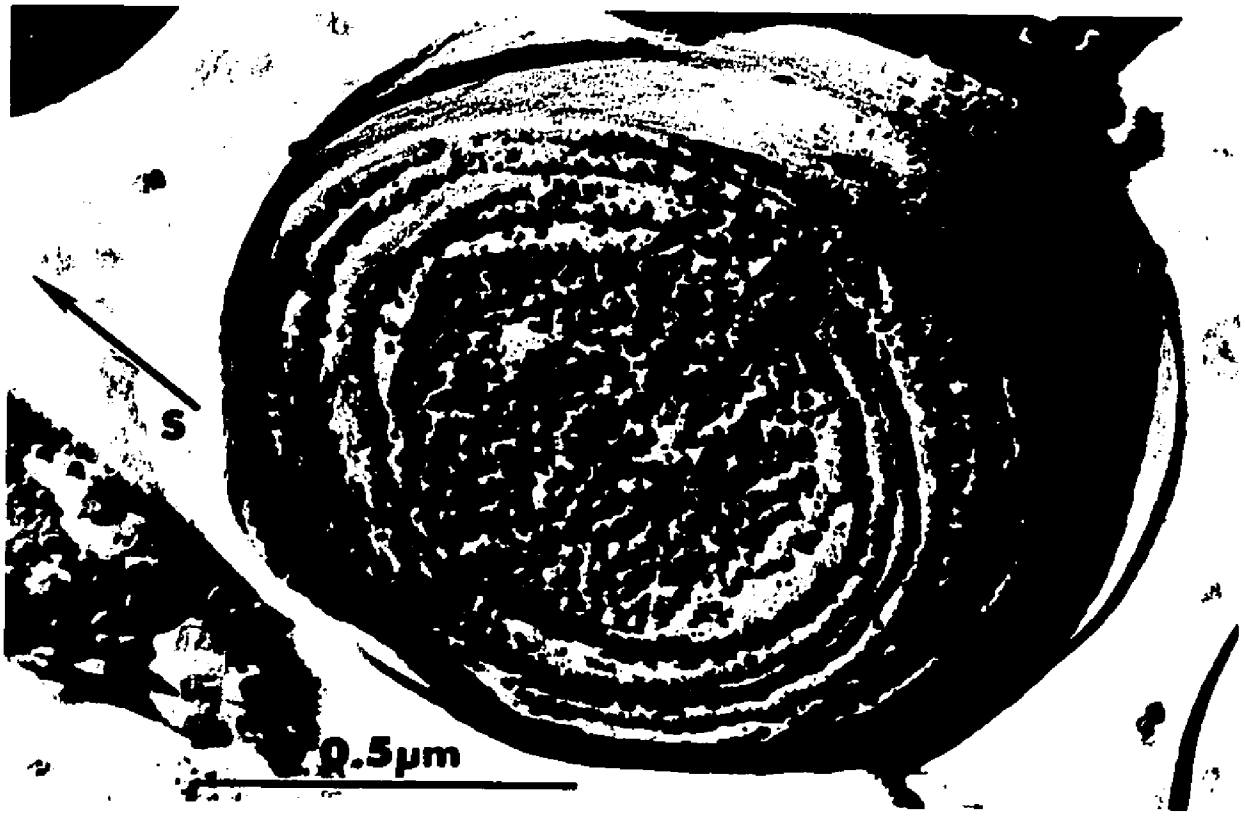

S
0.5μm

Figure 15. Electron micrograph of a freeze etch preparation of of *M. trichosporium* showing:

R = rod structure
S = direction of shadowing

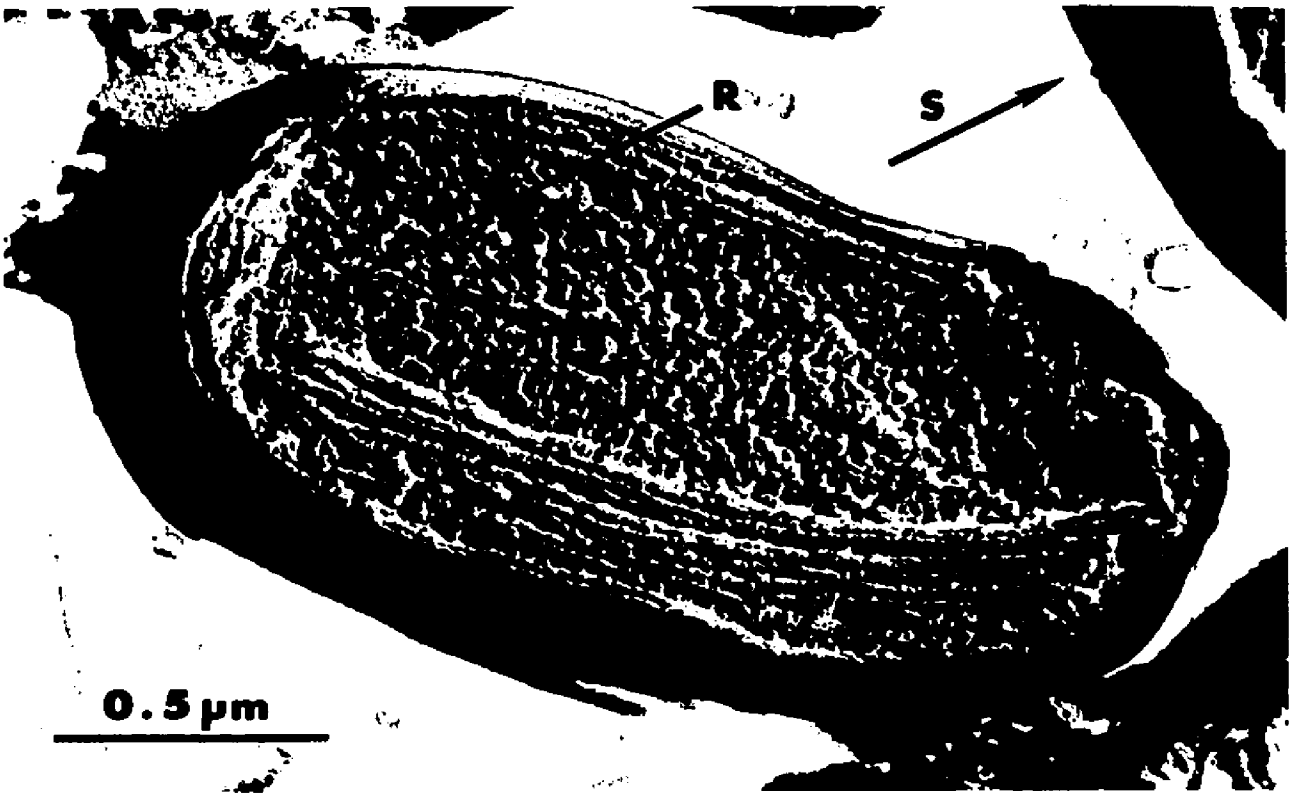

R
S
0.5 µm

Figure 16. Electron micrograph of a freeze etch preparation of *M. trichosporium* showing:

B = intercellular bridge
S = direction of shadowing

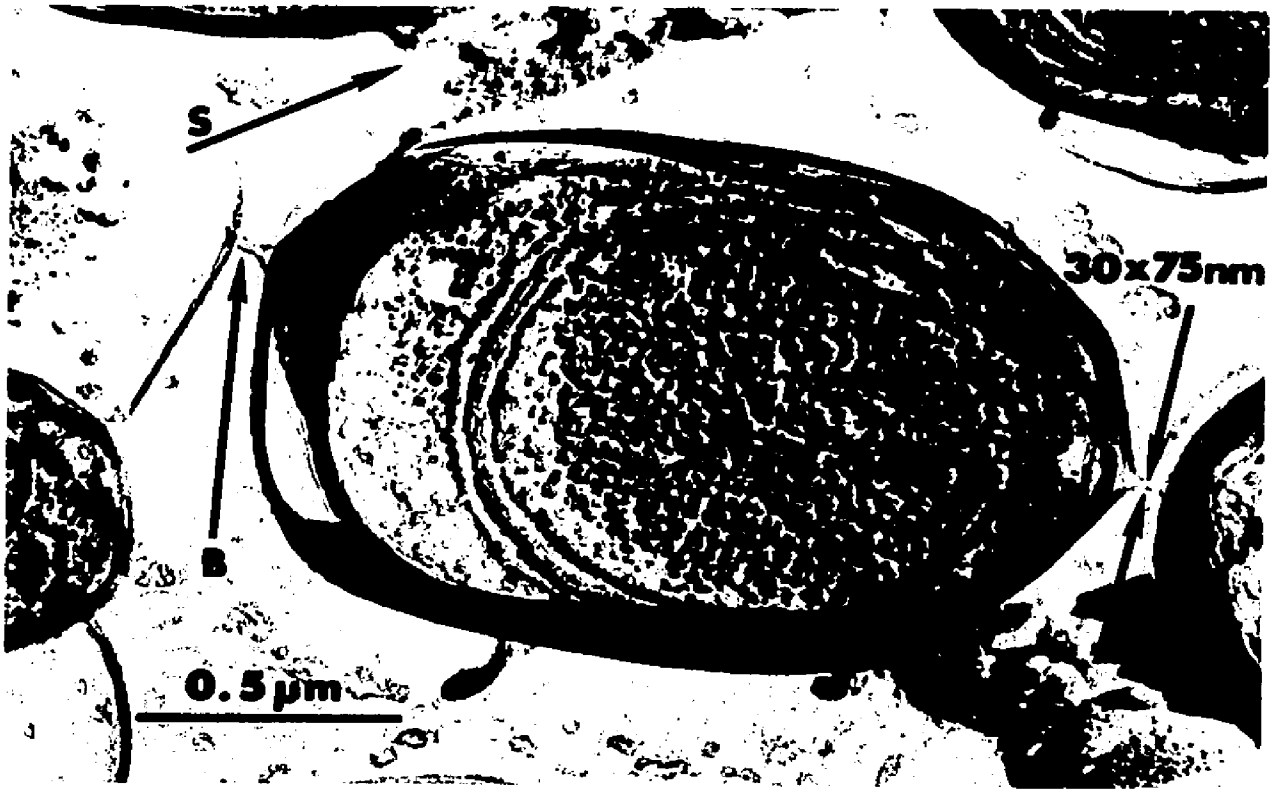

S
B
30x75nm
0.5 µm

Figure 17. Electron micrograph of a negatively stained sample of liquid M. trichosporium culture showing an intercellular bridge (B).

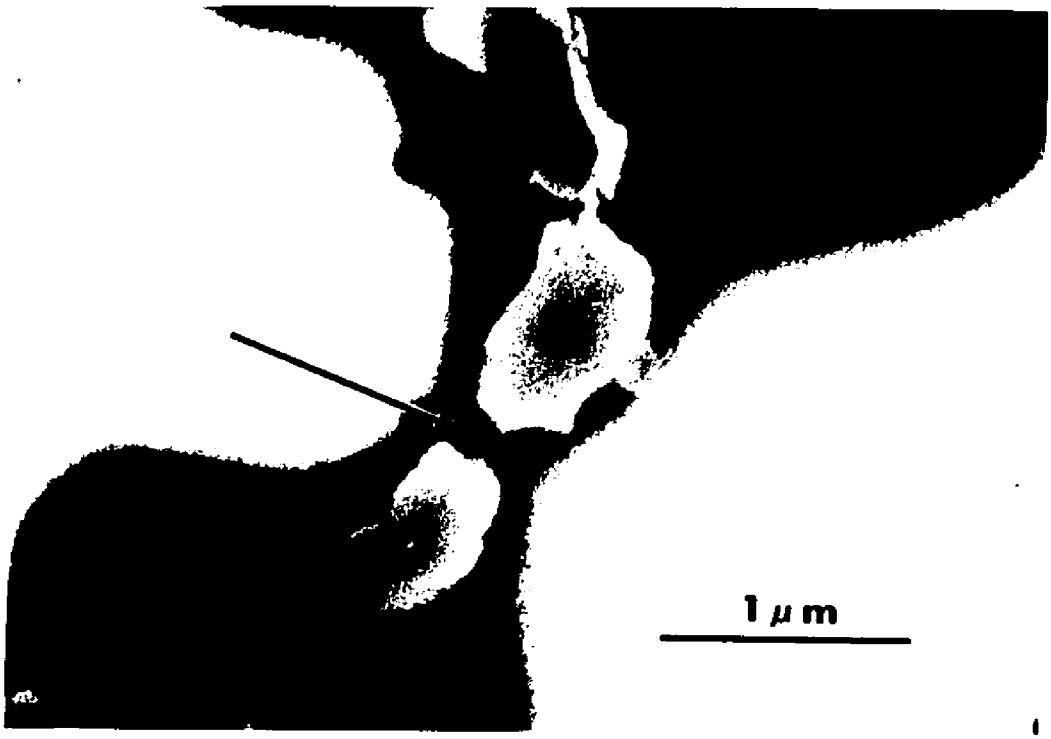

1 μm

Figure 18. Electron micrograph of a freeze etch preparation of M. trichosporium showing:

PHB = poly-beta-hydroxybutyrate
S = direction of shadowing


S
PHB

attached to them. Identification of these granules as PHB was based upon a similar appearance in other bacteria in addition to chemical identification, as will be explained in a subsequent section.

This peripheral arrangement of balloon-like vesicles is a Type II membrane system. The proposed organization of the Type II membranes in M. trichosporium is illustrated diagramatically in Figure 19

For comparison, a freeze etching and a thin section of M. methanica are shown in figures 20 and 21 respectively. These intracytoplasmic membranes are representative of a Type I system, a lamellar arrangement of stacked membrane vesicles lying in the center of the cell. Figure 21 reveals that this bacterium also accumulates PHB. Vesicular arrangement and membrane ultrastructure are shown in Figure 22. The membranes were 9.15 nanometers thick enclosing vesicles 15-18 nanometers in diameter. Figure 23 indicates that these Type I membranes are also passed along during cell division.

Both the Type I and the Type II membrane systems are very similar in appearance to the intracytoplasmic membranes of the photosynthetic bacteria (Oelze and Drews, 1972) and the nitrifying bacteria (Watson, 1965).

Figure 19. Illustration representing the proposed morphology of M. trichosporium.

OCW = outer cell wall
ICW = inner cell wall
PM = plasma membrane
IM = intracytoplasmic membrane
PHB = poly-beta-hydroxybutyrate

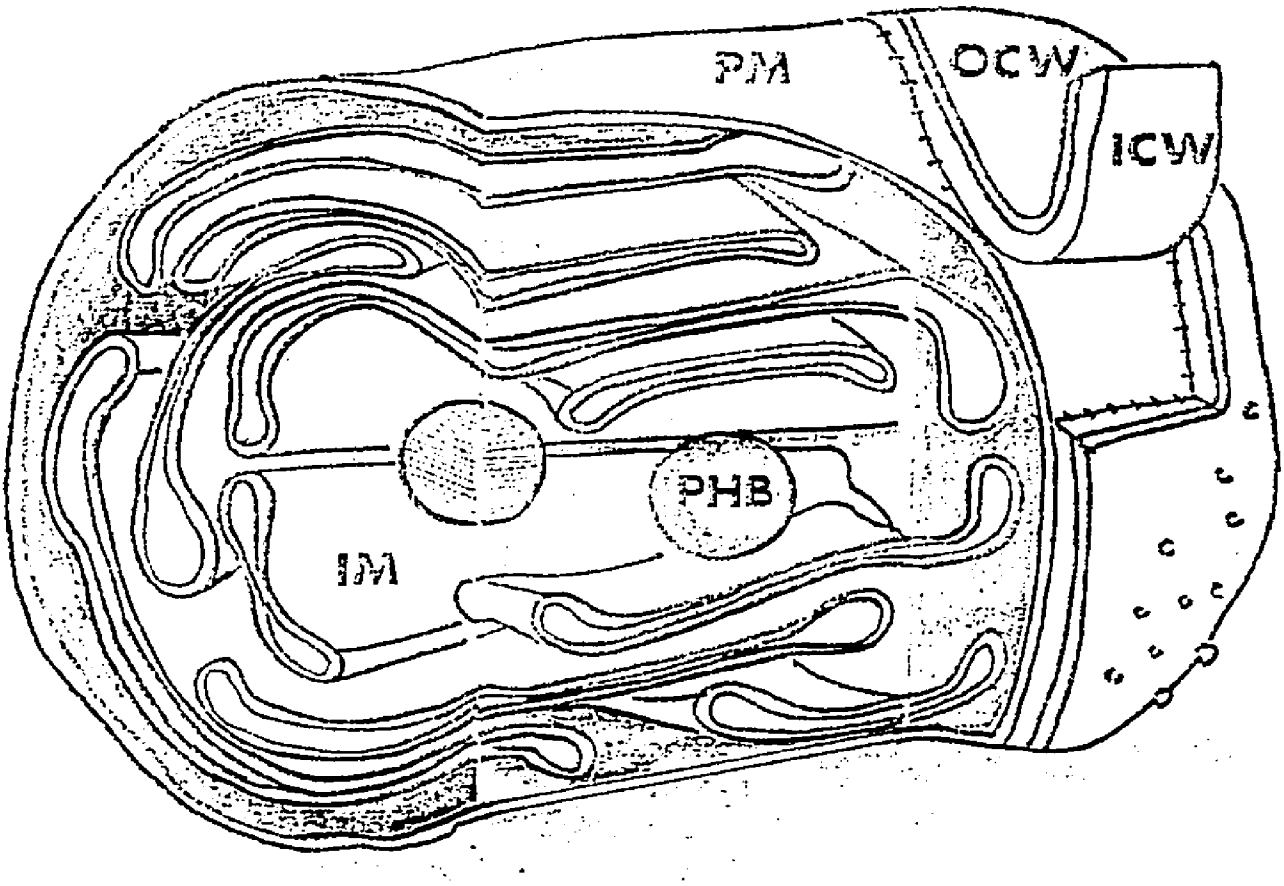

PM
OCW
ICW
PHB
IM

Figure 20. Electron micrograph of a freeze etch preparation of *M. methanica* showing Type I membranes.

IM = internal membranes
S = direction of shadowing


IM
S

Figure 21. Electron micrograph of a thin section preparation of *M. methanica* showing:

im = internal membranes
cm = cell membrane
cw = cell wall
phb = poly-beta-hydroxybutyrate
r = ribosomes

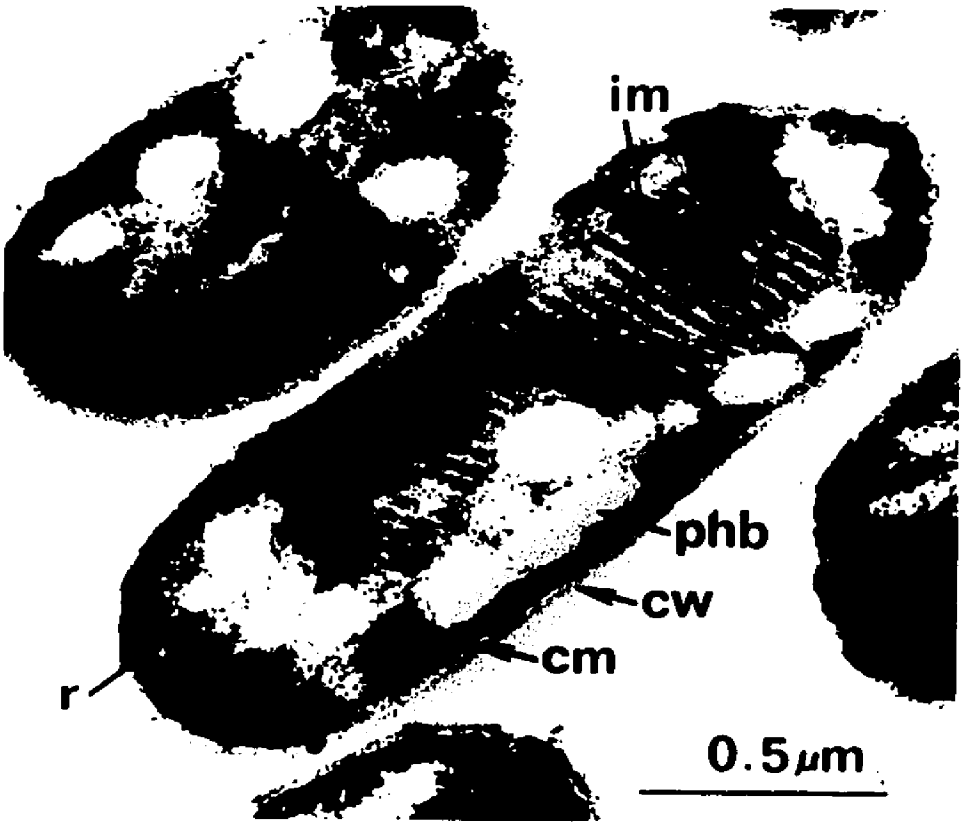

im
phb
cw
cm
r
0.5μm

Figure 22. Electron micrographs of thin sections of membranes in M. methanica showing:

A. Membrane dimensions
B. Comparison of cell membrane with internal membranes
   cm = cell membrane
   im = internal membranes
C. Membrane vesicles
   Arrows denote membrane continuity.
D. Membrane vesicles

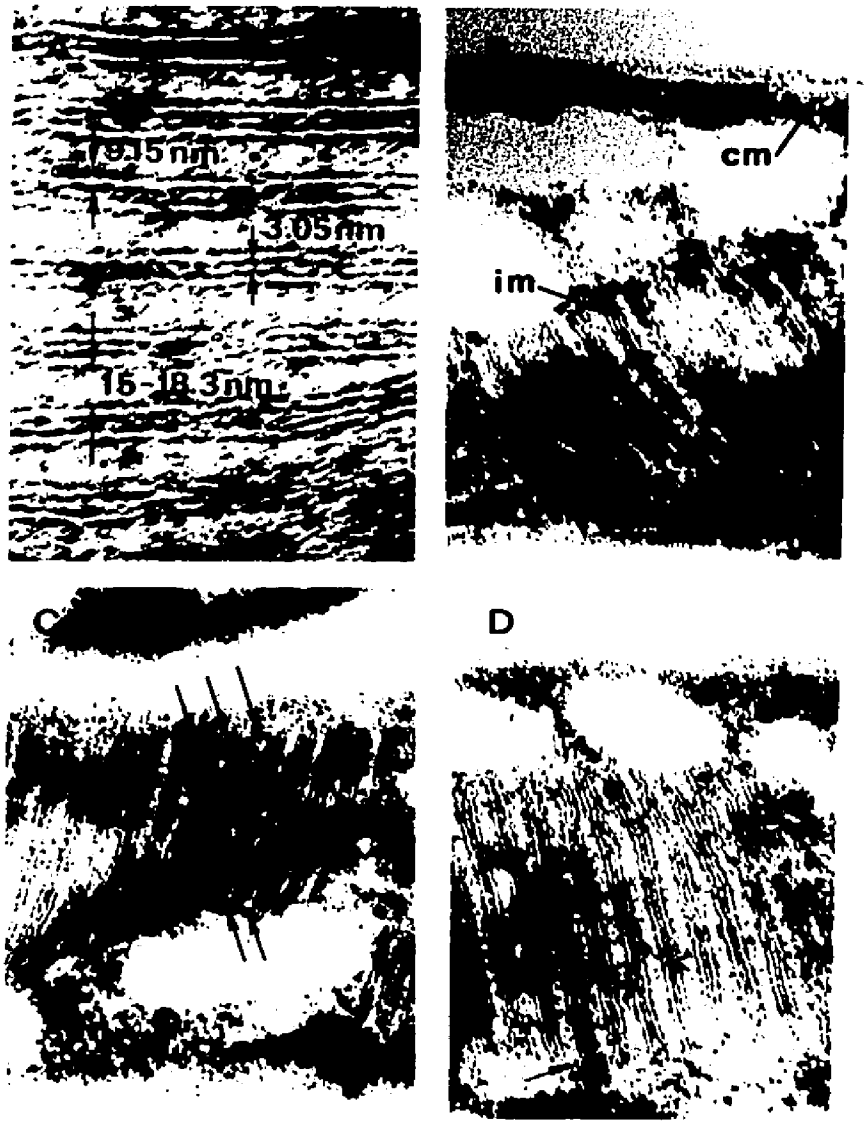
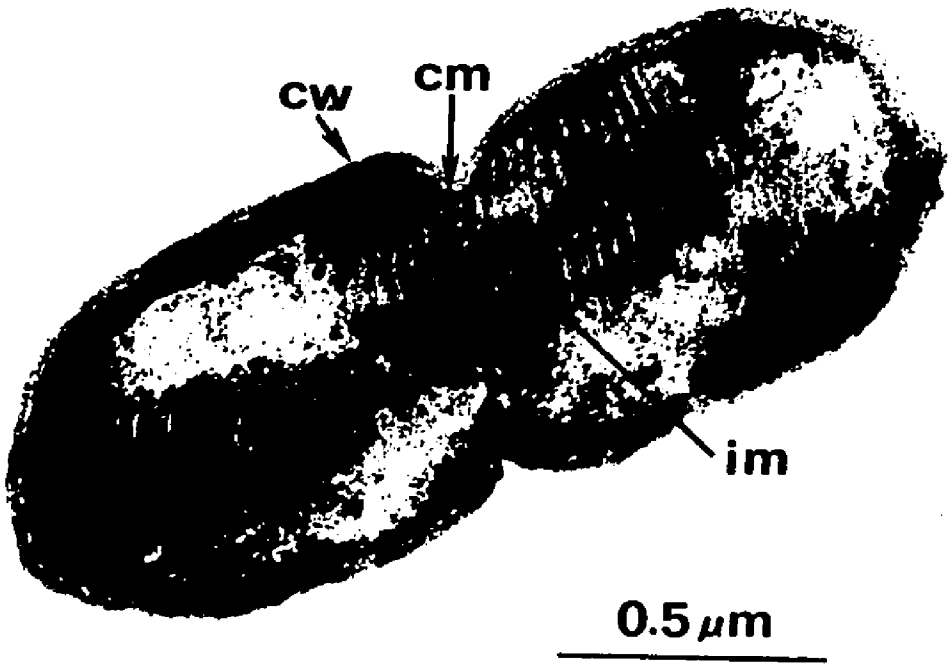

3.05nm
cm
im
C
D

Figure 23. Electron micrograph of a thin section preparation of M. methanica showing:

cm = cell membrane
cw = cell wall
im = internal membranes


cw
cm
im
0.5 μm

III. Isolation of Intracytoplasmic Membranes

An electron micrograph of negatively stained material from the 5P fraction is shown in Figure 24 and is typical of the material found in this fraction. This is indicative of the relatively heavy cell wall material that was in the 5P fraction. Also of significance is the fact that the cells were not grossly disrupted by this breakage procedure. There were large chunks of cell wall debris and PHB in the low speed fractions, but the relatively mild cell disruption evidenced in Figure 24 was the norm rather than the exception. The 20P fraction shown in Figure 25 shows that even at this moderate speed, the large pieces of cell debris had been previously sedimented. Figure 26 shows the 80P fraction containing relatively pure membrane material. Clumps seen in this fraction are characteristic of membrane material which forms aggregates due to hydrophobic areas in the membranes. Figure 27 shows a thin section of a membrane preparation revealing the typical ultrastructure of osmium fixed membranes.

Perhaps more valuable are the quantitative data listed in Table 4. These data show that most of the PHB sediments in the 5P fraction, a moderate amount in the 80S fraction, and little or none in the other fractions. The PHB in the 80S fraction could be due to buoyancy of the lipid material, but this seems unlikely since no granules appearing similar to PHB were observed in 80S negative stains. The possibility of fragments of PHB granules

Figure 24. Negative stain of the 5P differential centrifugation fraction of disrupted M. trichosporium cells.

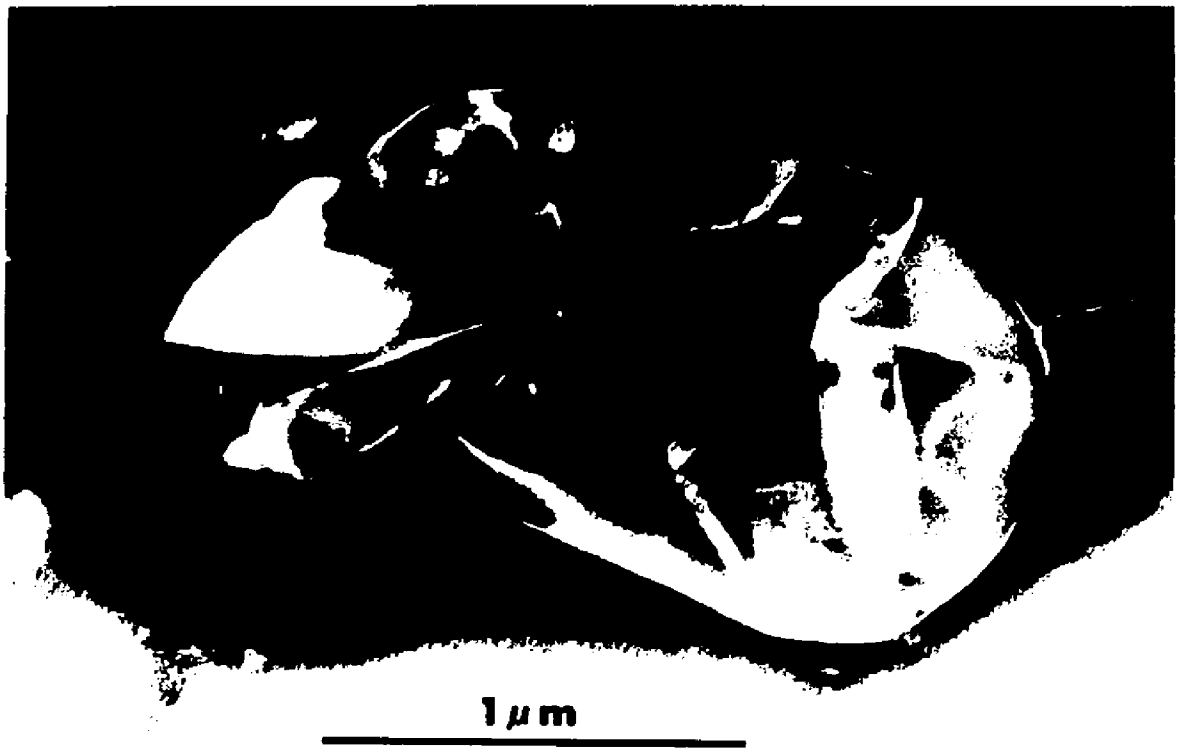

1 μm

Figure 25. Negative stain of the 20P differential centrifugation fraction of disrupted *M. trichosporium* cells.

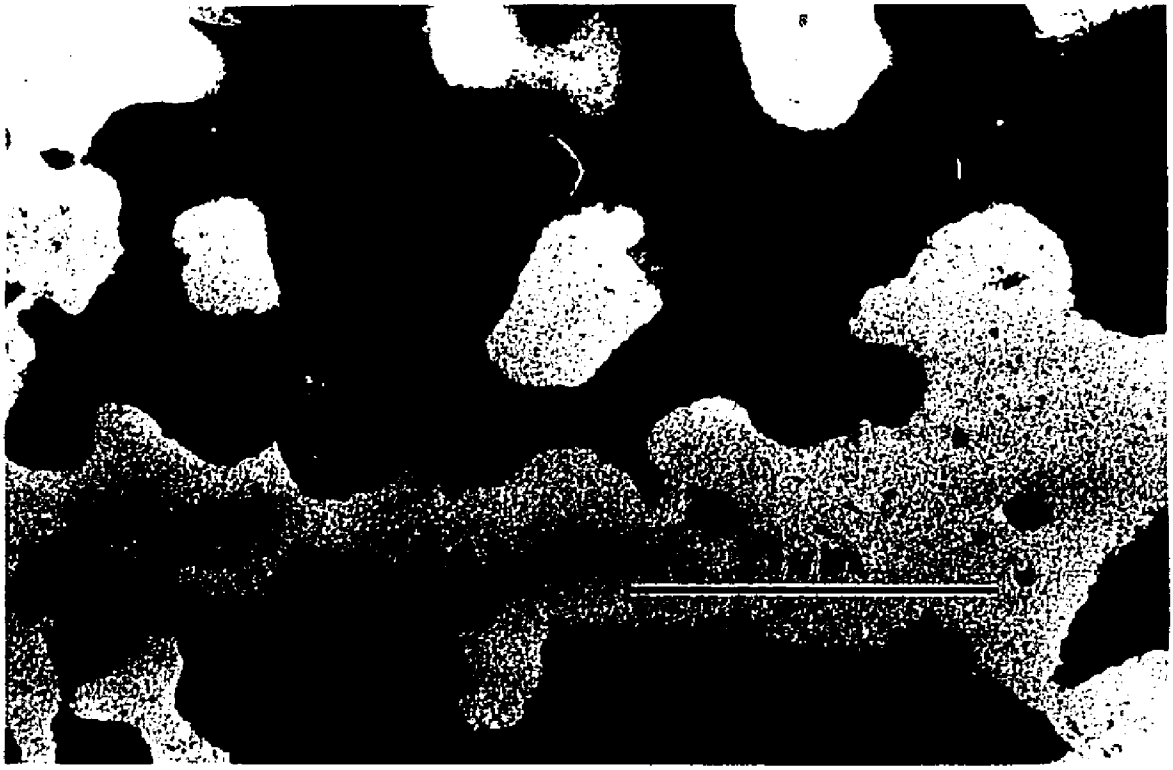


Figure 26. Negative stain of the 80P differential centrifugation fraction of disrupted M. trichosporium cells.

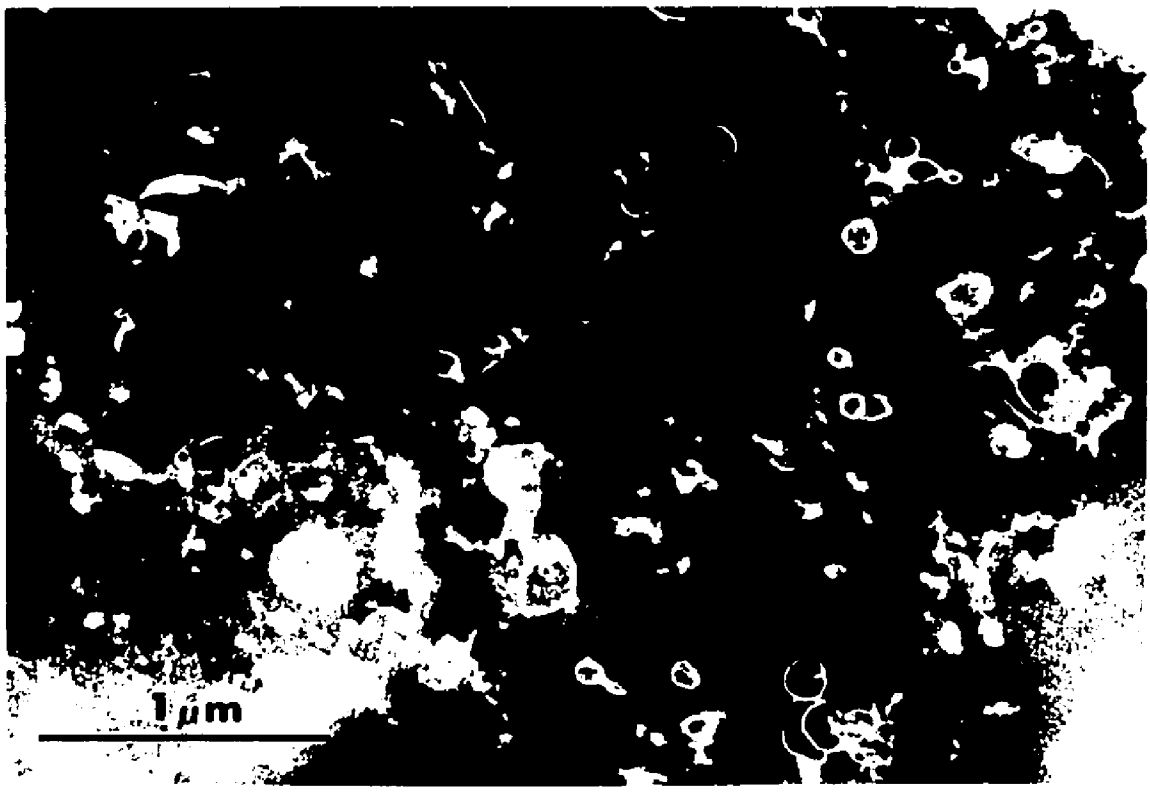

1 µm

Figure 27. Thin section of an inintracytoplasmic membrane preparation 20P, 40P, 80P, fractions from M. trichosporium.

0.5 μm

Table 4. Relative weight of protein, hexose, and PHB in various differential centrifugation fractions of broken M. trichosporium cells.

FRACTION	PROTEIN (mg)	HEXOSE (mg)	PHB (ug)	PROTEIN/HEXOSE
Broken cell	37.4	17.0	692	2.2
5P	17.2	7.57	567	2.3
10P	0.59	0.05	1.67	12
20P	0.50	0.05	0.93	10
40P	0.36	0.05	0.74	7.2
80P	0.24	0.05	0	4.8
80S	13.3	6.46	63.2	2.1

in the 80S fraction seems remote since such small quantities were observed in the 10P through 80P fractions. Another hypothesis that may explain this observation would be the presence of short chain partially polymerized and single molecules of beta-hydroxybutyrate in the 80S fraction (Eller and Lundgren, 1968).

The quantity of both protein and hexose decreased through the 80P fraction; this was expected since the total amount of material decreased. More important is the fact that the protein/hexose ratio increased in the 10P, 20P, 40P, and 80P fractions as compared with broken cells and the 5P fraction. Because cell walls contain large amounts of hexose while membranes are lipid-protein structures, this indicates membrane enrichment in the higher speed pellets. Since the cell wall material sedimented in the low speed fractions and because it has already been shown that in these bacteria the plasma membrane is attached to the cell wall, the higher speed pellets represent relatively purified intracytoplasmic membranes. Thus, the plasma membrane-cell wall association of Gram negative bacteria that has so bothered membrane investigators in the past has in this study been an advantage making it possible to obtain preparations of intracytoplasmic membranes.

IV. Lipid Characterization

Because of its simplicity and better yields, the Folch extraction was used throughout this study rather than the Bligh and Dyer technique.

The methanol precipitate of the total lipid extract was identified as poly-beta-hydroxybutyrate. Results of an infrared spectrophotometric comparison of this precipitate with purified PHB are shown in Figure 28. The dominant characteristic of this spectrum is the large ester peak at 1725 cm^{-1}. These spectra both compare well with the published Bacillus megaterium PHB spectra of Blackwood and Agnes (1957).

The results of a gravimetric analysis of whole cell and 20P membrane lipids are listed in Table 5. Only about 9.2% of the cell dry weight was extracted as lipid while 27.9% of the membrane dry weight extracted as lipid. This lipid enrichment in the membrane fraction would be expected since membranes are basically lipid-protein structures. This compares very favorably with previous reports of 21% lipid in inner mitochondrial membrane and 25% lipid in Gram positive bacteria plasma membrane (Guidotti, 1972a). However, these in turn are very different from the 42% lipid in liver cell membranes, 43% lipid in erythrocyte membranes, and 75% lipid in myelin (Guidotti, 1972a). Myelin may have increased lipid because of

Figure 28. Infrared spectra of (upper spectrum) methanol precipitate from total lipids of M. trichosporium and (lower spectrum) purified PHB.

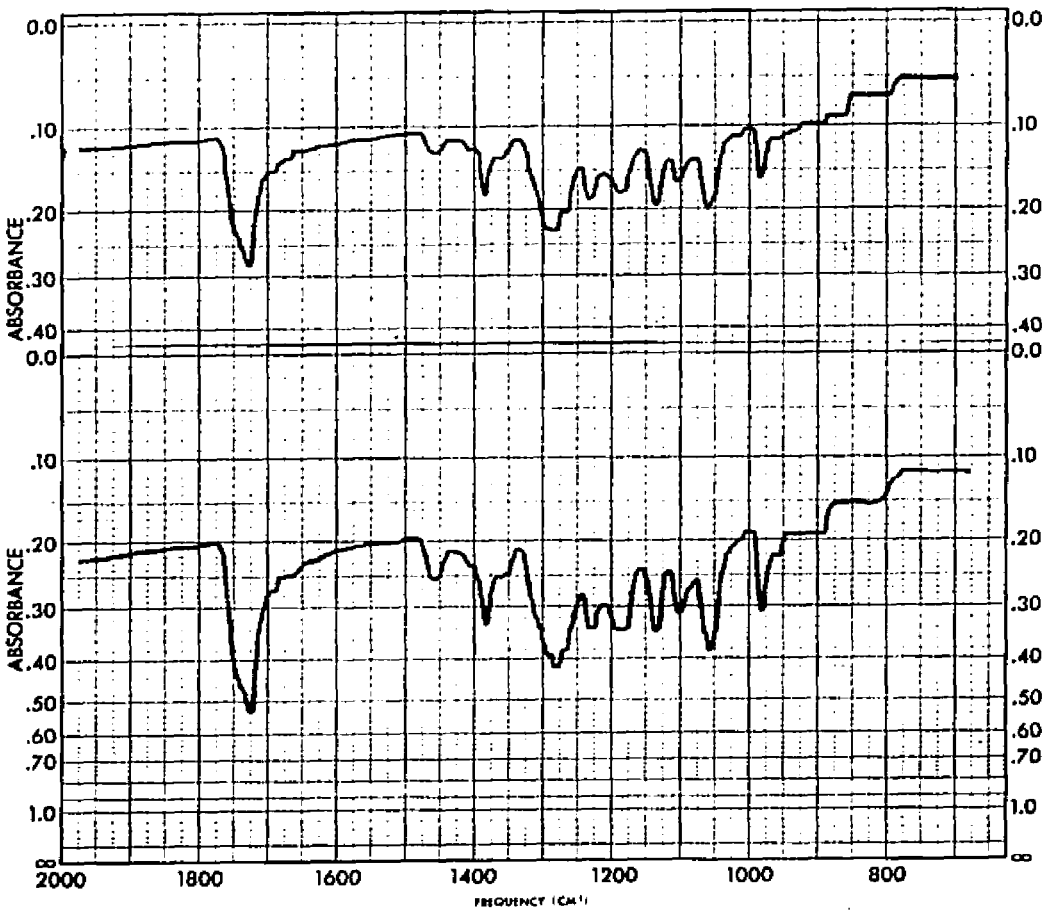

ABSORBANCE
0.0
.10
.20
.30
.40
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
2000
1800
1600
1400
1200
1000
800
FREQUENCY (CM⁻¹)

Table 5. Gravimetric analysis of lipid components of whole cells and membranes.

	Weight Extracted From Cells (mg)	Weight Extracted From Membranes (mg)
PHB	18.9	0.1
simple lipid	28.0	3.8
phospholipid	24.7	19.6
total	71.6	23.5
	% Dry Weight of Cells	% Dry Weight of Membranes
PHB	2.4	0.11
simple lipid	3.6	4.5
phospholipid	3.2	23.3
total	9.2	27.9
	% Dry Weight of Total Cell Lipids	% Dry Weight of Total Membrane Lipids
PHB	26.4	0.4
simple lipid	39.1	16.2
phospholipid	34.5	83.4

its requirement for insulation. The relatively low lipid in these intracytoplasmic membranes probably indicates the important enzymatic role (ergo, more protein) performed by these internal membranes. The relatively large amount of PHB found in whole cells (i.e. 26.1% of total lipid) indicates that this compound must play an important role in the physiology of this organism. Presumably, the trace of PHB in the membrane sample was due to contamination. The percent phospholipid was greatly increased from the 34.5% of whole cell lipids to 83.1% in the membrane sample. This would be expected since phospholipids almost exclusively comprise bacterial membrane lipids. This value is higher than the 67% phospholipid reported for erythrocyte membranes (Guidotti, 1972a), but it is not unusual because eucaryotic membranes contain steroids while procaryotic membranes usually do not. Because of the relatively large amount of phospholipid and because of their importance in membrane structure, the phospholipids were investigated more intensely in this study.

The results of the simple lipid separation are listed in Table 6. Spot #1 (simple lipid #1 or SL-1) was identified as hydrocarbon, SL-2 was identified as triglyceride, SL-3 was identified as free fatty acid, SL-4 was identified as 1,3-diglyceride, and SL-5 was tentatively identified as 1,2-diglyceride based upon the fact that it is a common lipid component and is known to migrate slightly behind 1,3-diglyceride in this solvent.

Table 6. R_f Values of cell and membrane simple lipids and standards on silica gel TLC in hexane-diethyl ether-acetic acid.

Spot #	R_f
1	.95
2	.66
3	.44
4	.36
5	.27

Standard	R_f
free fatty acid	.44
triglyceride	.64
1,3-diglyceride	.36
hydrocarbon	.96

Quantitative data from the simple lipid analysis are shown in Table 7. The fact that whole cell simple lipids were different from membrane simple lipids would be expected, but the magnitude of the difference was surprising. Even though simple lipids are relatively minor membrane components, their composition in membranes was found to be very different from that of whole cells. The predominance of triglycerides in whole cells is not unusual since these simple lipids are commonly reported in bacteria although usually in lower quantities (O'Leary, 1967). The prevalence of free fatty acids in membrane simple lipids is not unusual either because of the amphipathic nature of these molecules. With their part hydrophilic and part hydrophobic structure, fatty acids fit nicely into membrane structure as currently theorized. However, the large amount of hydrocarbon found in the membrane fraction is difficult to explain. Whatever role hydrocarbons play in membrane structure, they must be isolated in the hydrophobic regions of the membrane interior.

Five phospholipids were detected by TLC. The same five spots in the same ratios were detected whether analyzing whole cells or membranes. This was expected since nearly all cellular phospholipids are in membranes (O'Leary, 1967). A summary of the TLC data for membrane phospholipids is shown in Table 8. By comparing these

Table 7. Quantitative analysis of simple lipids from whole cells and membranes.

	Percent of Total Simple Lipid	
	Whole Cells	Membranes
hydrocarbon	6.8	43.5
triglycerides	73.7	6.7
diglycerides	2.5	0
free fatty acids	17	49.8

Table 8. Summary of silica gel TLC data concerning membrane phospholipids.

	Spot # 1	2	3	4	5
R_f in CMW[1]	.53	.39	.32	.21	.18
R_f in BAW[2]	.57	.38	.48	.48	.23
ninhydrin	-	+	-	+	-
iodine	+	+	+	+	+
plasmalogen	-	-	-	-	-
orcinol	-	-	-	-	-
Goswami	+	+	+	+	+
Dragendorf	-	-	-	-	-

1 Chloroform:methanol:water (65:25:4)

2 Butanol:acetic acid:water (60:60:20)

data with the R_f values of phospholipid standards listed in Table 9, the phospholipids were identified. Phospholipid #1 (PL-1) was identified as diphosphatidyl glycerol; PL-2 was identified as phosphatidyl ethanolamine; PL-3 was identified as phosphatidyl glycerol; PL-4 was identified as phosphatidyl serine; PL-5 was identified as phosphatidyl choline. Since PL-5 was not Dragendorf positive, it could be a lysocompound. However, in view of the unreliability of the Dragendorf test and since this spot ran with phosphatidyl choline in 2 solvents, this was ample evidence to identify PL-5 as phosphatidyl choline.

Results of the argentation TLC are listed in Table 10. Development in the second direction through silver nitrate did not yield any further separation of the individual spots. This indicated homogeneity of the spots. If there were different species varying in fatty acid saturation, they would have separated in the second direction. Diphosphatidyl glycerol was not present in sufficient quantity to be detected on this plate. Further evidence of phospholipid homogeneity will be described later.

Table 9. R_f values of phospholipid standards.

	R_f	
	CMW[1]	BAW[2]
diphosphatidyl glycerol	.53	.57
phosphatidyl ethanolamine	.39	.38
phosphatidyl serine	.22	.45
phosphatidyl choline	.19	.23
phosphatidyl glycerol	.33	.49

1 Chloroform:methanol:water (65:25:4)

2 Butanol: acetic acid: water (60:20:20)

Table 10. R_f values for membrane phospholipids on argentation TLC plates.

	R_f	
	1st	2nd
phosphatidyl ethanolamine	.39	.43
phosphatidyl glycerol	.32	.43
phosphatidyl serine	.21	.44
phosphatidyl choline	.18	.58

The results of the phospholipid hydrolysis experiments are listed in Table 11. Only the hydrolysis products of phosphatidyl ethanolamine were detected, presumably because of their quantity and ninhydrin reactivity. The water soluble products ran exactly with the appropriate standards and verify the identification of PL-2 as phosphatidyl ethanolamine.

Table 12 shows the results of the glycol analysis with PL-3; PL-2 and silica gel were used as controls. A thousand times more formaldehyde was produced with PL-3 than with either of the other samples. This supports the identification of PL-3 as phosphatidyl glycerol.

An autoradiogram of a thin layer plate after separation of phospholipids labeled with P^{32} is shown in Figure 29. This autoradiogram verifies the previous identification of 5 phospholipid spots. Relative abundance is also obvious in the autoradiogram with phosphatidyl ethanolamine and phosphatidyl glycerol comprising the bulk of the phospholipids. The radiochromatogram scanner tracing of this TLC plate is shown in Figure 30, and the relative peak areas from this tracing are listed in Table 13. Scintillation counts of spots scraped from these plates are listed in Table 14.

Table 11. R_f values for the hydrolysis products of phosphatidyl ethanolamine compared to standards.

A. Water soluble mild alkaline hydrolysis product.

	R_f
glycerophosphorylethanolamine	.74
hydrolysis product A	.72
glycerophosphorylserine	.39

B. Acid hydrolyzed product A.

	R_f
ethanolamine	.25
hydrolysis product B	.25
serine	.22

Table 12. Formaldehyde quantitation after periodate oxidation of phospholipids.

	Color	Formaldehyde (micromoles)
Blank	brown	less than 0.01
PL-2	brown/purple	0.01
PL-3	purple/black	10

Figure 29. Autoradiogram of P^{32} labeled phospholipids separated by TLC.

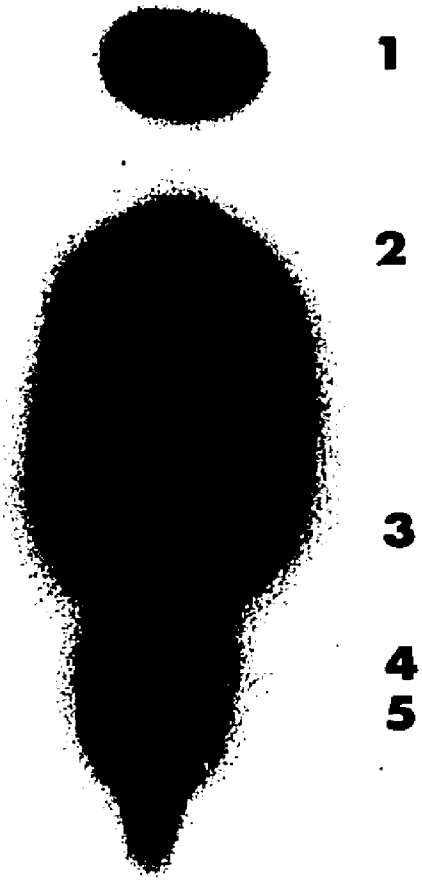

1
2
3
4
5

Figure 30. Radiochromatogram scanner tracing of P^{32} labeled phospholipids separated by TLC.

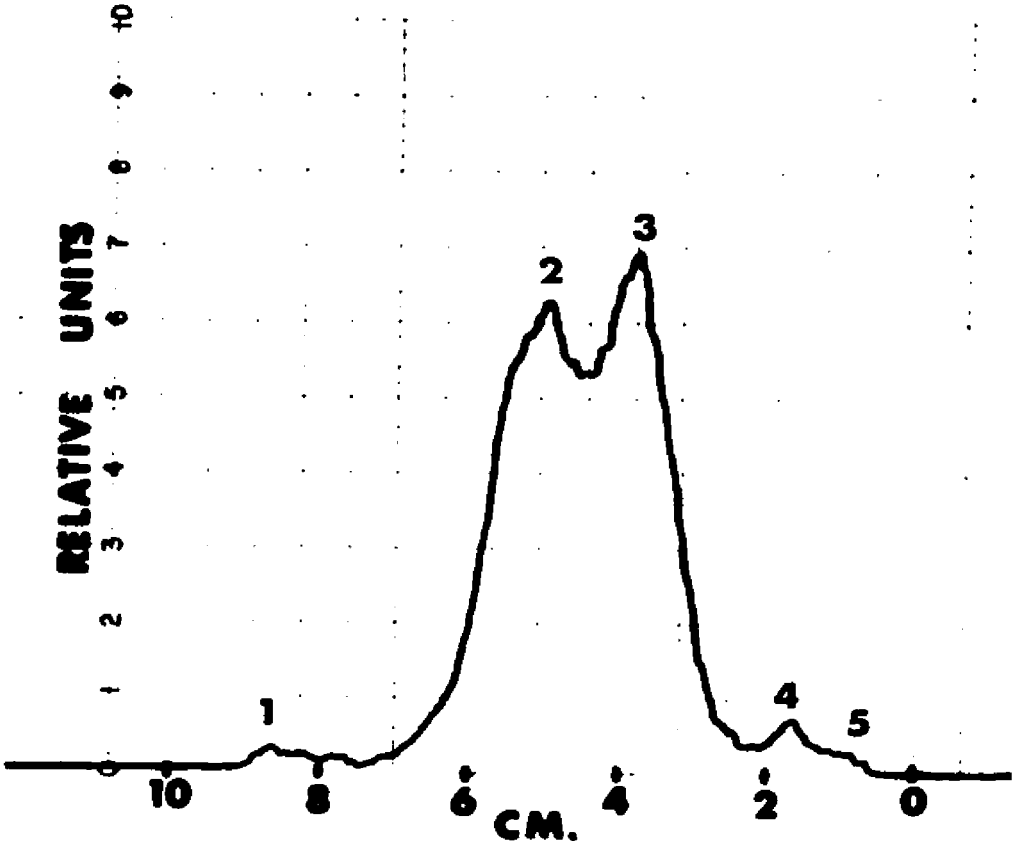

RELATIVE UNITS
1
2
3
4
5
10
8
6
4
2
0
CM.

Table 13. Relative peak areas from radiochromatogram scanner tracing of P^{32} labeled phospholipids separated by TLC.

	Relative Area	% Total Activity
diphosphatidyl glycerol	5	2
phosphatidyl ethanolamine	106	44
phosphatidyl glycerol	118	49
phosphatidyl serine	8	3
phosphatidyl choline	4	2

Table 14. Scintillation spectrophotometric quantitation of P^{32} labeled phospholipids scraped from a TLC plate.

	C.P.M.	% Activity
diphosphatidyl glycerol	2,956	1
phosphatidyl ethanolamine	150,198	37
phosphatidyl glycerol	229,583	57
phosphatidyl serine	16,862	4
phosphatidyl choline	2,419	1

From these data, it was observed that 93-94% of the total phospholipid consisted of phosphatidyl ethanolamine and phosphatidyl glycerol. The rest of the phospholipids made up about 6% of the total. It is not unusual for these 2 phospholipids to comprise 70-85% of total phospholipids in bacteria, but the magnitude of predominance found in this study is higher than normally observed. Several structurally and physiologically similar bacteria also have this higher percentage of phosphatidyl ethanolamine and phosphatidyl glycerol. For example, among the photosynthetic bacteria, these two phospholipids constitute 100% of the total in Chromatium, 98% of the total in Rhodopseudomonas gelatinosa, 90% of the total in Rhodopseudomonas spheroides, and 86% of the total in Rhodospirillum rubrum (Oelze, 1972). Within the nitrifying bacteria, these two phospholipids account for 95% of the total phospholipids in both Nitrocystis oceanus and Nitrosomonas europea (Hagen, 1966). The significance of this phospholipid composition is unknown, but the similarities with the other bacteria with intracytoplasmic membranes are obvious.

The results of the fatty acid analysis of these phospholipids is shown in Table 15. In view of the reduction of the major peak after bromine addition, it was identified as monounsaturated 18:1 fatty acid as opposed to 18:0 which also comes off in the

Table 15. Fatty acid compositions of the phospholipids of *M. trichsporium*.

	Percent Total		
	16:0	16:1	18:1
diphosphatidyl glycerol	0	13	87
phosphatidyl ethanolamine	0.2	10.9	88.9
phosphatidyl glycerol	1	16	83
phosphatidyl serine	0	13	87

	Ratio 16:0 to 16:1 to 18:1
fatty acids before bromine	190:50:1
after bromine	4:1:1

vicinity of this very large peak. These results are similar to the report of Smith and Ribbons (1970) concerning phospholipids from Methanomonas methanoxidans where over 90% of the esterified fatty acid was 18:1. Lack of diversity of fatty acids and a preponderance of 1 particular fatty acid is more characteristic of eucaryotic phospholipids than bacterial phospholipids which usually contain a variety of fatty acids. For example, White (1968) described 36 relatively evenly distributed fatty acids ranging from 12-22 carbons in the phospholipids of Haemophilus parainfluenzae. Once again of interest are similarities between M. trichosporium and the photosynthetic and nitrifying bacteria. The ammonia oxidizing bacteria show a preponderance of 16:1 esterified fatty acid on the phospholipids while the nitrite oxidizing bacteria have mostly 18:1 fatty acid (Blumer et al., 1969). Among the photosynthetic bacteria, Rhodomicrobium vannielii has 90% 18:1 fatty acid in its phospholipids, Rhodopseudomonas capsulata has 94.6% 18:1 fatty acid, and Rhodopseudomonas spheroides has 76.8% 18:1 fatty acid in its phospholipids (Oelze, 1972). Similarity of fatty acid composition of phospholipids could be advantageous to an organism by facilitating transfer of components among the various phosphatides. This could be especially important to an organism

whose metabolism is very dependent upon complex membrane systems and may explain the lack of diversity among the phospholipid fatty acids in M. trichosporium. The reason for the prepond-erance of 18:1 fatty acid is more difficult to explain. This is the longest fatty acid (with respect to the fatty acids normally found in bacteria) that would remain liquid at normal physiological temperatures. However, why only certain organisms have large amounts of 18:1 fatty acid is unknown. While the morphological and biochemical similarities among the methane oxidizing bacteria, photosynthetic bacteria, and nitrifying bacteria are obvious and interesting, their explanation and possible relationship remains to be elucidated.

V. Membrane Protein Characterization

The suggestion that membranes tend to have less polar amino acids than other proteins was made by Vanderkooi and Capaldi (1972). Vanderkooi and Capaldi used a relatively complex procedure for their analyses which required knowledge of absolute amino acid composition for computations. Therefore, one of the goals in this study was to investigate the proposal of Vanderkooi and Capaldi by simpler and faster techniques. The percent polarity

for 100 nonmembrane proteins whose composition is listed in the Handbook of Biochemistry (Saber et al., 1970) was compiled in Figure 31. The average polarity of these nonmembrane proteins was 33%. These computations were done to serve as the norm for subsequent amino acid analyses.

The observed R_f values for 19 amino acids in the previously described silica gel TLC system are listed in Table 16. The apolar amino acids had R_f values greater than 0.5 while the polar amino acids were less than 0.5. This convenient separation made it possible to perform this amino acid polarity study. The results of the polarity analyses are shown in Table 17. The whole cell and 80S samples both fall near the mean polarity of the nonmembrane protein analysis. All the membrane fractions had increased levels of apolar amino acids and exhibited a very low polarity as compared to the nonmembrane proteins in Figure 31. This can be explained by the hypothesis that if membranes contained excesses of polar amino acids, they would become solubilized in water and could not function as a barrier in an aqueous environment.

Figure 31. Histogram showing (A) polarity of 2 membrane associated enzymes and (B) distribution of polarity among 100 nonmembrane proteins.

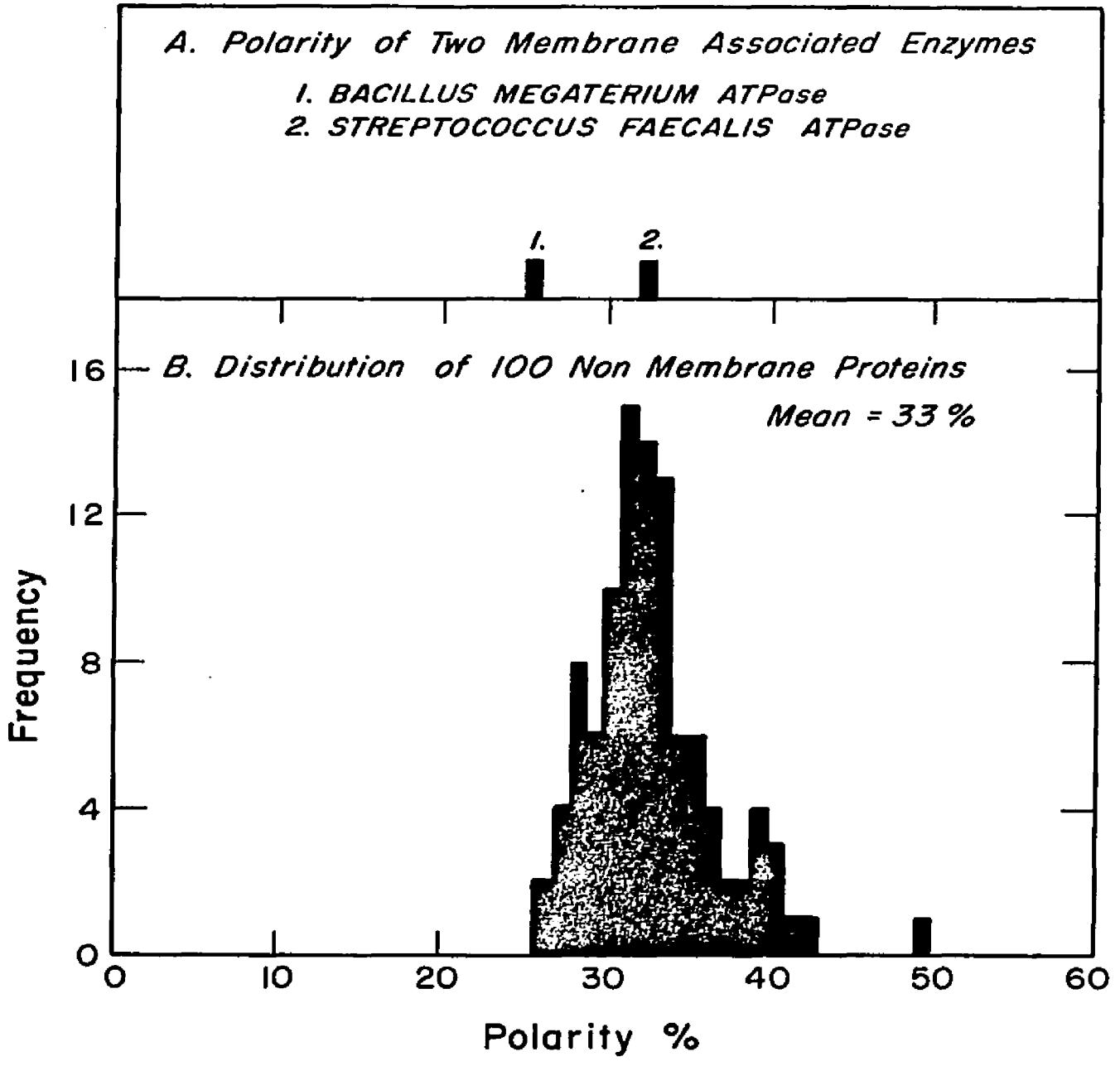

A. Polarity of Two Membrane Associated Enzymes
1. BACILLUS MEGATERIUM ATPase
2. STREPTOCOCCUS FAECALIS ATPase
1.
2.
B. Distribution of 100 Non Membrane Proteins
Mean = 33 %
Frequency
0
4
8
12
16
0
10
20
30
40
50
60
Polarity %

Table 16. R_f values for amino acids on silica gel TLC plates developed with ethanol and ammonium hydroxide.

Amino Acid	R_f
phenylalanine	.75
leucine	.75
isoleucine	.75
tryptophane	.74
methionine	.70
valine	.69
alanine	.67
threonine	.66
histidine	.64
serine	.62
glycine	.62
proline	.59
hydroxy proline	.59
cystine	.58
cysteine	.58
glutamic acid	.49
aspartic acid	.33
lysine	.25
arginine	.22

Table 17. Polarity of amino acids in various protein samples from M. trichosporium.

Sample	Mole % Apolar	Polar
whole cells	68.4	31.6
10P	71.6	28.4
20P	73.9	26.1
80P	71.4	28.6
80S	67.2	32.8

A photograph of the stained membrane protein polyacrylamide gels is shown in Figure 32, and the approximate molecular weights of the proteins are listed in Table 18. Guidotti (1972b) observed 9 proteins in human erythrocyte membranes ranging in molecular weight from 25,000 to 250,000. Two of these proteins (47,000 and 80,000) had identical molecular weights to M. trichosporium intracytoplasmic membrane proteins. The intracytoplasmic membranes analyzed in this study contained 5 major proteins ranging in molecular weight from 47,000 to 180,000 which is similar to the distribution reported by Guidotti (1972b) with erythrocyte membranes. Due to the problem of obtaining solubilized membrane protein in sufficient quantities to detect on a gel, the proteins observed in this study probably represent only the major membrane proteins. Membrane enzymes, for example, that may be required in catalytic amounts probably wouldn't have been detected by this technique.

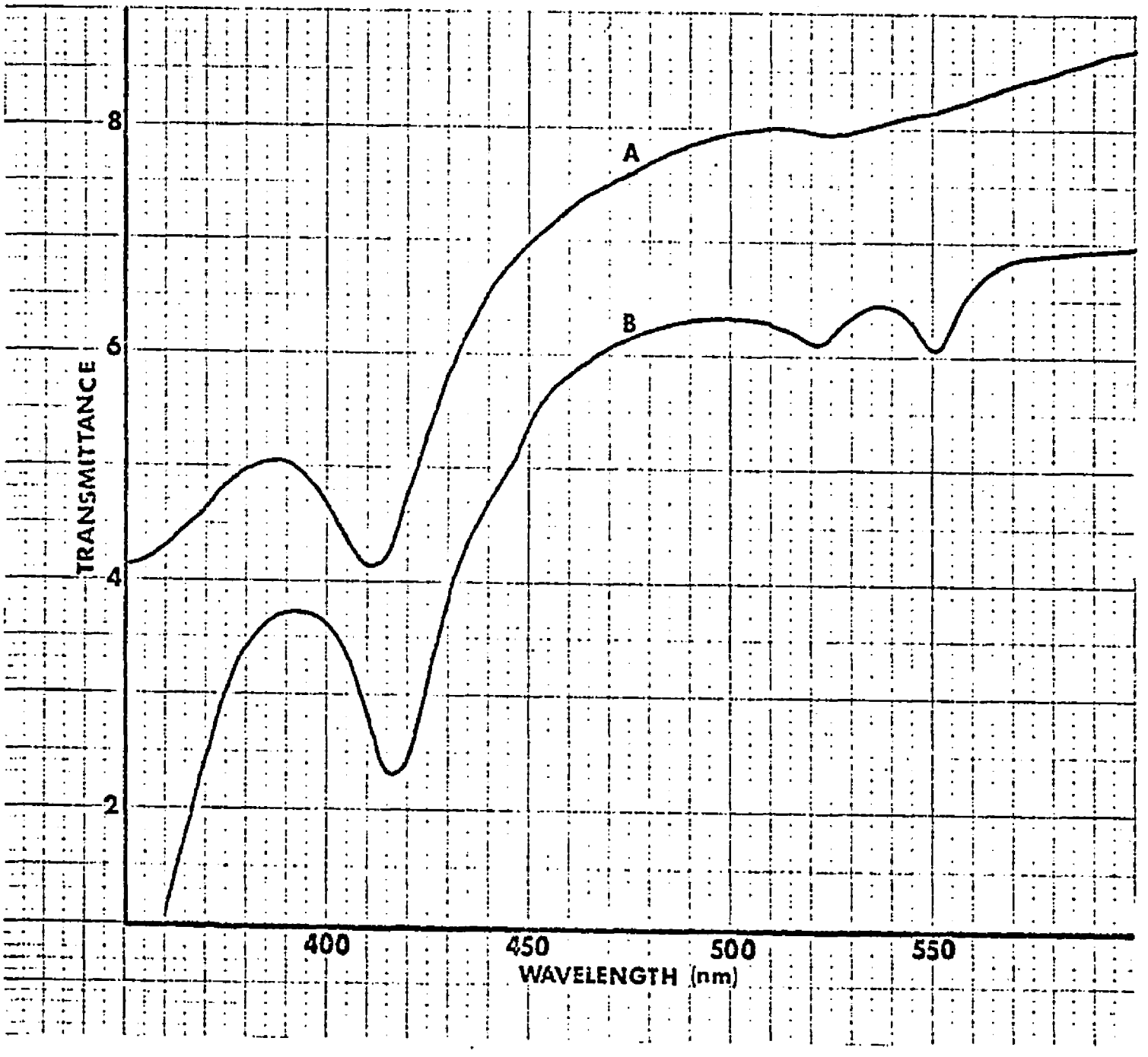
Results of a spectrophotometric examination of an 80P membrane preparation are shown in Figure 33. The oxidized spectrum absorbs maximally at 410 nanometers, and the reduced spectrum has absorbance peaks at 416, 522, and 550 nanometers, which is the spectrum of a C type cytochrome. The shift of the gamma or Soret peak to a longer wavelength, i.e. 410 to 416 nanometers, and the appearance of the alpha and beta peaks, i.e. 550 and 522 nanometers, when reduced are typical

Figure 32. Photograph of electrophoretic polyacrylamide gels containing (A) bovine serum albumin and membrane proteins, and (B) bovine serum albumin.

Table 18. Migration distances and molecular weights of membrane proteins and bovine serum albumin standard as related to polyacrylamide gel electrophoresis.

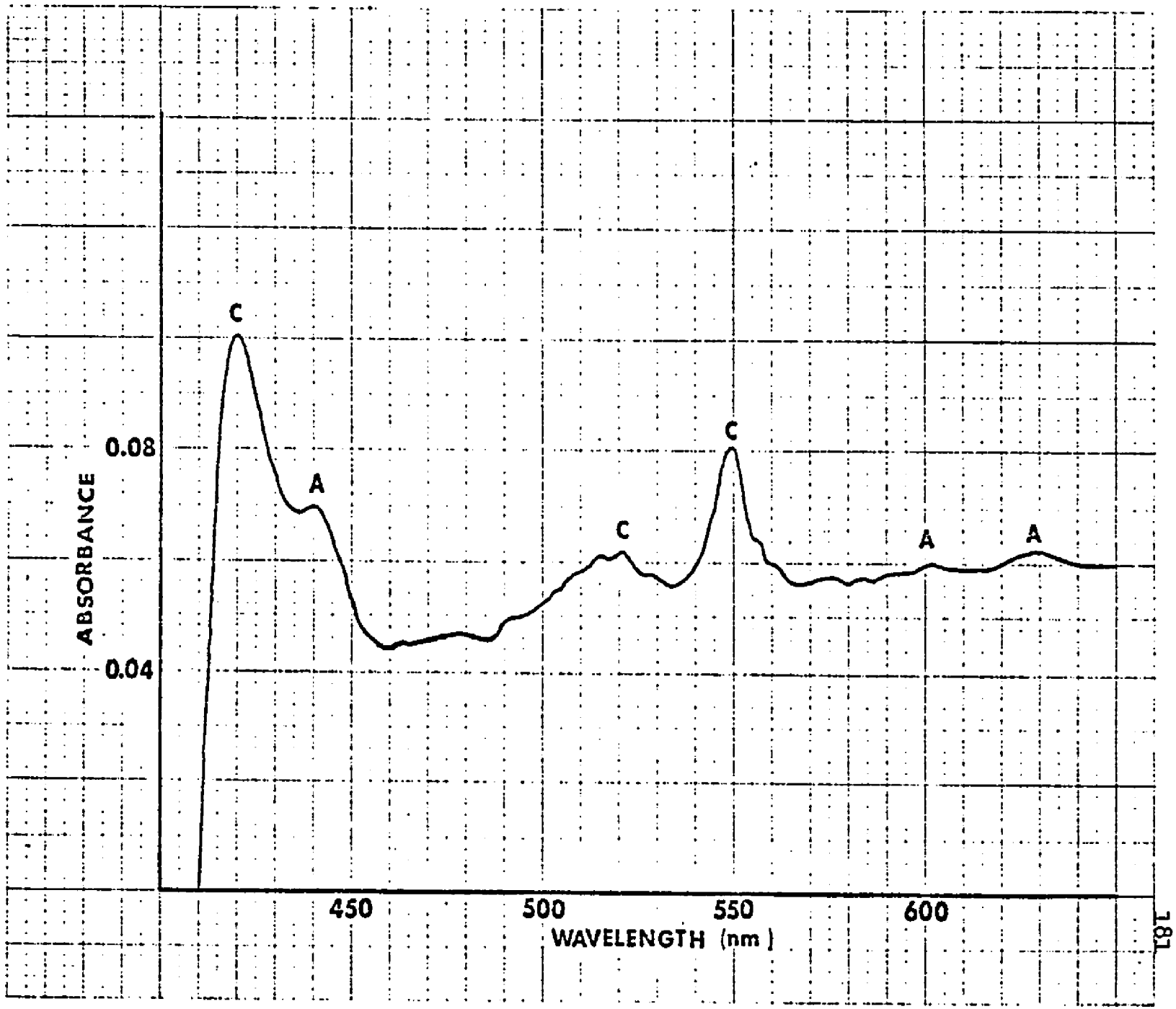
	Migration Distance (mm)	Molecular Weight
BSA (monomer)	22	60,000
BSA (dimer)	13	120,000
membrane protein 1	25	47,000
membrane protein 2	18	80,000
membrane protein 3	12	125,000
membrane protein 4	9	160,000
membrane protein 5	7	180,000

Figure 33. Absorption spectra of a 80P membrane preparation from M. trichosporium when (A) oxidized and (B) reduced.


TRANSMITTANCE
8
6
4
2
A
B
400
450
500
550
WAVELENGTH (nm)

of C cytochromes. More specifically, these data correspond to cytochrome C_2. Previously reported cytochromes categorized as C_2 include the cytochrome C from denitrifying bacteria with reduced absorption peaks at 550, 522, and 416 nanometers and oxidized Soret peak at 410 nanometers, and a C cytochrome from Rhodospirillum rubrum which absorbs maximally when reduced at 550, 521, and 416 nanometers and at 409 nanometers when oxidized (Mahler and Cordes, 1966). Once again, similarities among this methane oxidizing bacterium, the photosynthetic, and the nitrifying bacteria are evident. Upon close examination of the reduced spectrum, a shoulder can be seen at 445 nanometers. All peaks were enhanced by using oxidized membranes in the reference cuvette against reduced membranes in the sample cuvette. This difference spectrum is shown in Figure 34. The small shoulder was much better resolved in this difference spectrum and absorbed maximally at 445, 600, and 630 nanometers. This is characteristic of cytochrome A. C cytochromes have a reduction potential around 220 millivolts and A cytochromes are around 300 millivolts. Assuming these two components function in an electron transport system, the flow of electrons would be from cytochrome C_2 to cytochrome A to oxygen. There are probably other carriers in this electron transport chain between cytochrome C_2 and NAD, however, these remain to be found. The fact that

Figure 34. Difference spectrum of oxidized 80P membranes versus reduced 80P membranes.


C
A
C
C
A
A
0.08
0.04
ABSORBANCE
450
500
550
600
WAVELENGTH (nm)

oxidation of NADH and the intermediates in the methane oxidation pathway can be coupled to cytochrome reduction is illustrated in Figure 35. The addition of methanol , formaldehyde, formate, and NADH to a 20S fraction resulted in cytochrome C_2 reduction as evidenced by the shift in the Soret peak and the appearance of the alpha and beta peaks. The results with methane were inconclusive but it certainly didn't reduce the cytochrome C_2 like the other compounds. This is consistent with the earlier observation that only 1 oxygen atom is consumed per oxidative step in the oxidation of methane by M. trichosporium. If the initial step in methane oxidation, which is probably a monooxygenase, resulted in cytochrome reduction, then 2 oxygen atoms would be required for this step.

VII. Enzyme Assays

In order to avoid confusion, the Commission on Enzymes of the International Union of Biochemistry (CEIUB) numbers, systematic names, trivial names, and reactions of the enzymes assayed are listed in Table 19. Results of the various enzyme assays are shown in Figures 36-42. The initial velocities and activities of the enzymes are listed in Table 20.

Figure 35. Absorption spectra of 20S centrifugation fractions. Spectra represent (A) oxidized spectrum, (B) sample plus methane, and (C) sample plus methanol, formaldehyde, formate, or NADH.

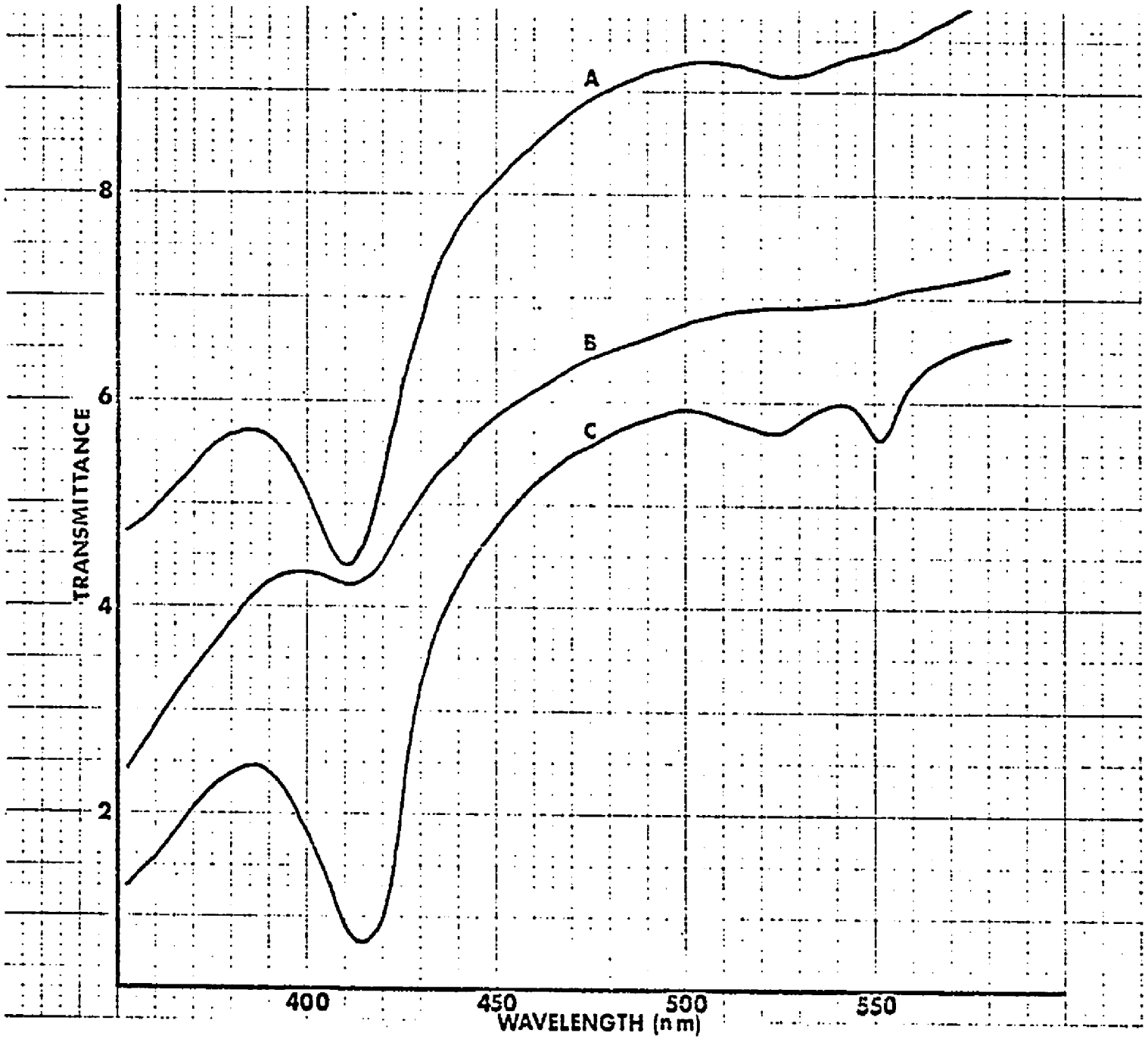

A
B
C
8
6
4
2
TRANSMITTANCE
400
450
500
550
WAVELENGTH (nm)

Table 19. Enzyme nomenclature listing (1) CEIUB number (2) systematic name (3) trivial name (4) reaction.

A.
1. 2.1.2.1
2. L-serine:tetrahydrofolate 5,10-hydroxymethyl transferase
3. serine trans hydroxymethylase
4. L-serine + tetrahydrofolate = glycine + 5,10-methylene THF

B.
1. 1.1.1.1
2. alcohol:NAD oxidoreductase
3. alcohol dehydrogenase
4. alcohol + NAD = aldehyde or ketone + NADH

C.
1. 1.2.1.3
2. formaldehyde:NAD oxidoreductase
3. formaldehyde dehydrogenase
4. formaldehyde + NAD = formate + NADH

D.
1. 1.2.1.2
2. formate:NAD oxidoreductase
3. formate dehydrogenase
4. formate + NAD = CO_2 + NADH

E.
1. 1.6.2.1
2. NADH:cytochrome C oxidoreductase
3. cytochrome C reductase
4. NADH + oxidized cytochrome C = NAD + reduced cytochrome C

F.
1. 3.6.1.3
2. ATP phosphohydrolase
3. ATPase
4. ATP + H_2O = ADP + orthophosphate

(continued next page)

(Table 19 continued)

G.
1. 1.2.1.29
2. D-glycerate:NAD oxidoreductase
3. glycerate dehydrogenase or hydroxypyruvate reductase
4. glyceric acid + NAD = NADH + hydroxypyruvate

H.
1. 1.1.1.31
2. 3-hydroxybutyrate:NAD oxidoreductase
3. beta-hydroxybutyrate dehydrogenase
4. 3-hydroxybutyrate + NAD = acetoacetate + NADH

Figure 36. Graphs showing activity of serine transhydroxymethylase.

○ supernatent activity
□ membrane activity
▩ NADPH control, membranes
● no substrate

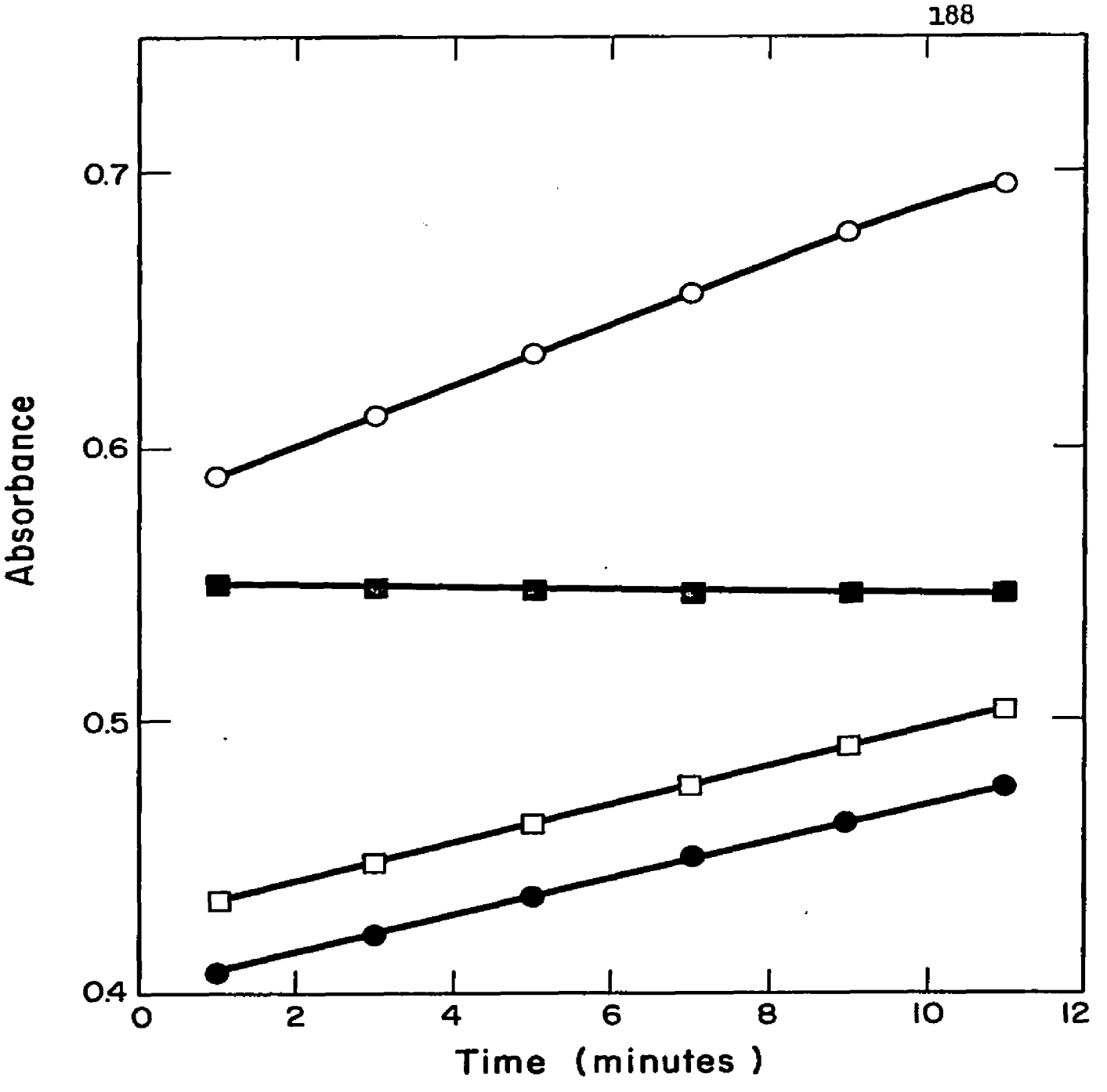
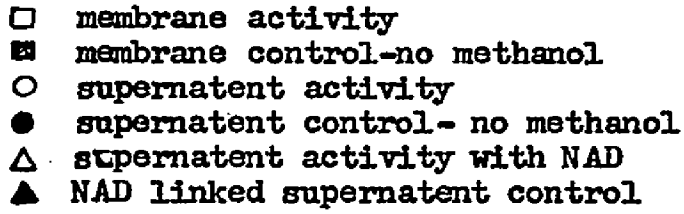

0.7
0.6
0.5
0.4
Absorbance
0
2
4
6
8
10
12
Time (minutes)

Figure 37. Graphs showing methanol dehydrogenase activity.



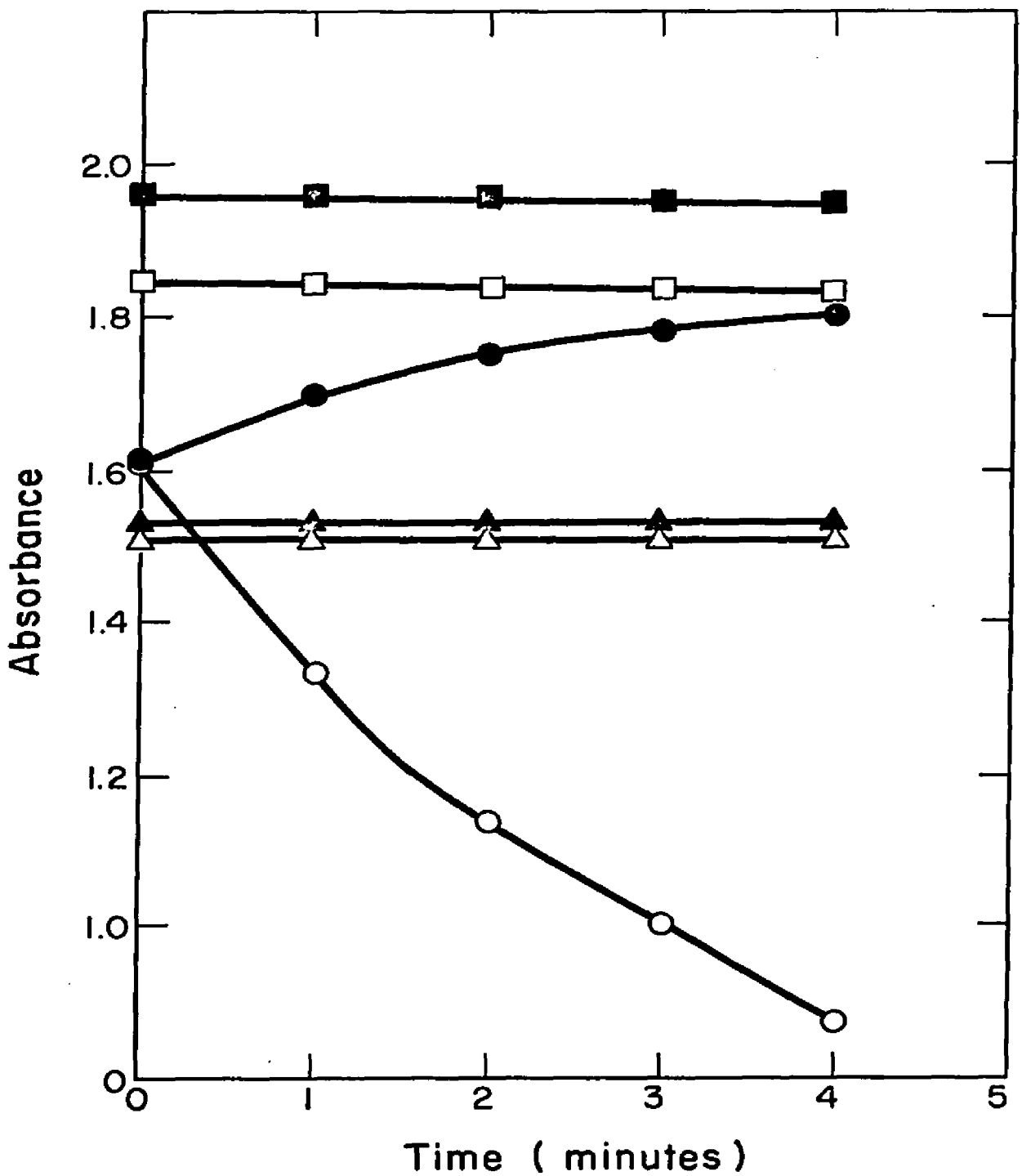

2.0
1.8
1.6
1.4
1.2
1.0
0
Absorbance
0
1
2
3
4
5
Time (minutes)

Figure 38. Graphs showing formaldehyde dehydrogenase activity.

□ membrane activity
▩ membrane control- no formaldehyde
○ supernatent activity
● supernatent control- no formaldehyde

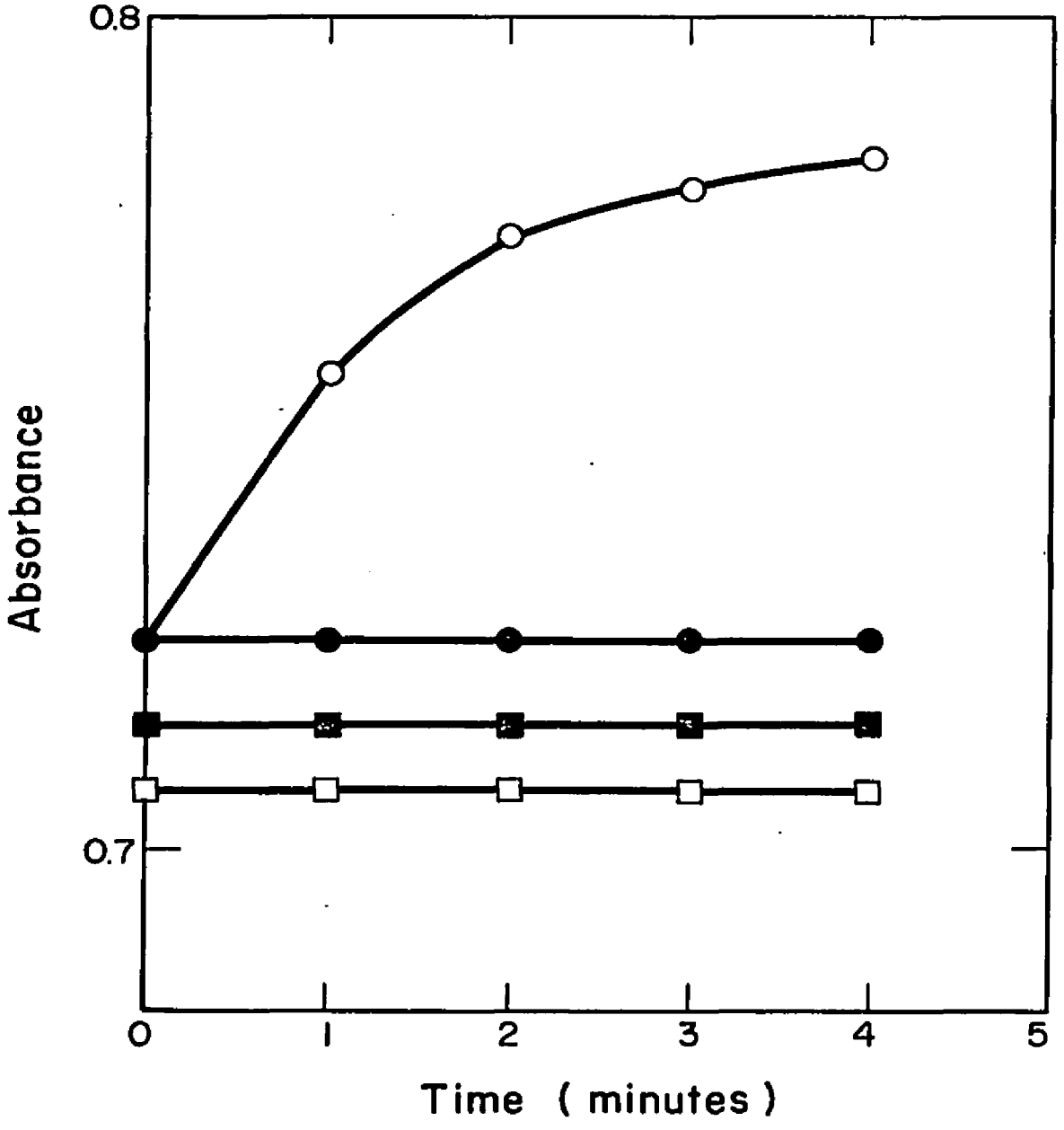

0.8
0.7
Absorbance
0
1
2
3
4
5
Time (minutes)

Figure 39. Graphs showing formate dehydrogenase activity.

■ membrane activity and control
○ supernatent activity
● supernatent control- no formate

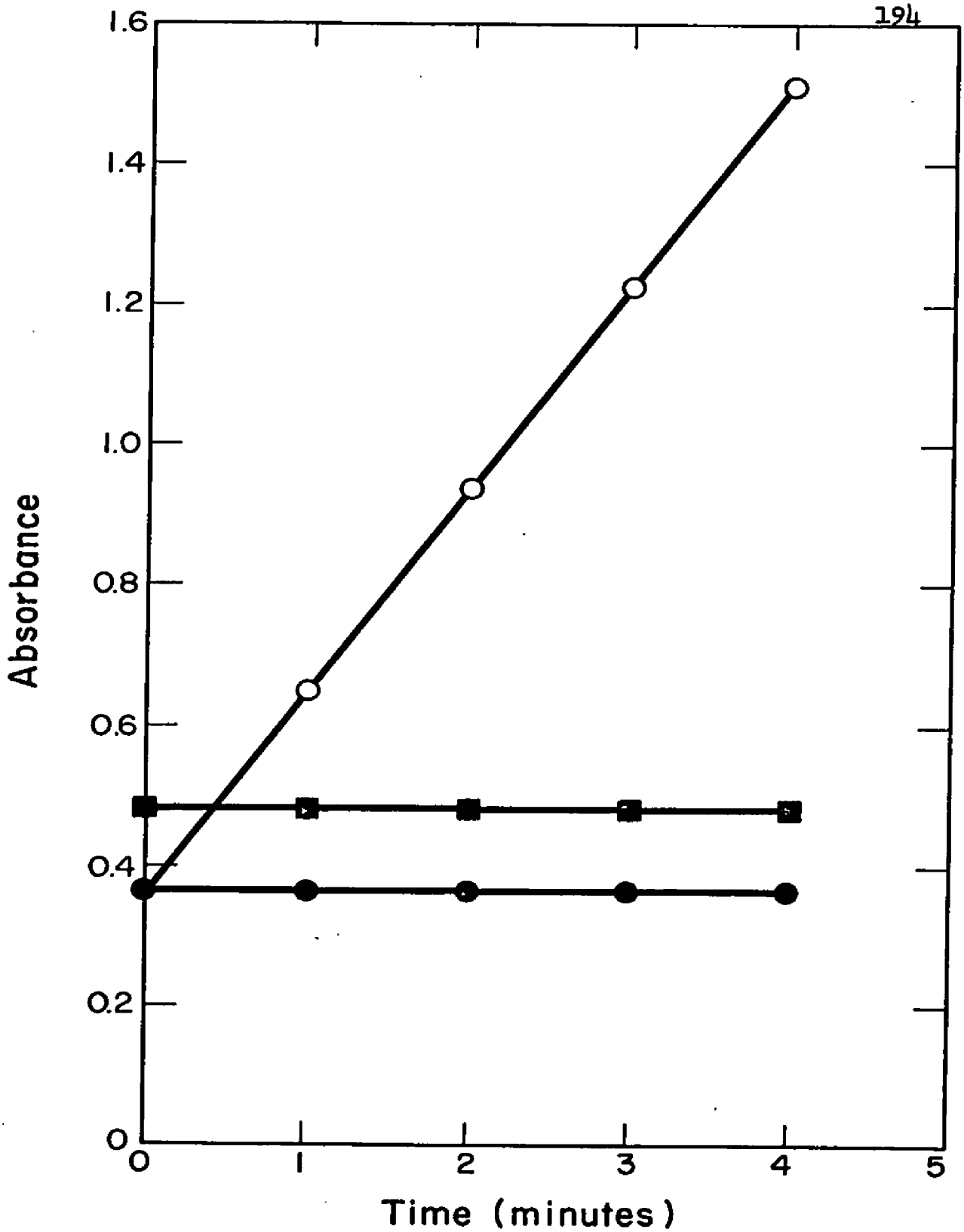
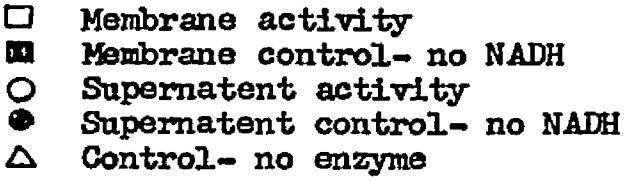

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Absorbance
0
1
2
3
4
5
Time (minutes)

Figure 40. Graphs showing cytochrome C reductase activity.



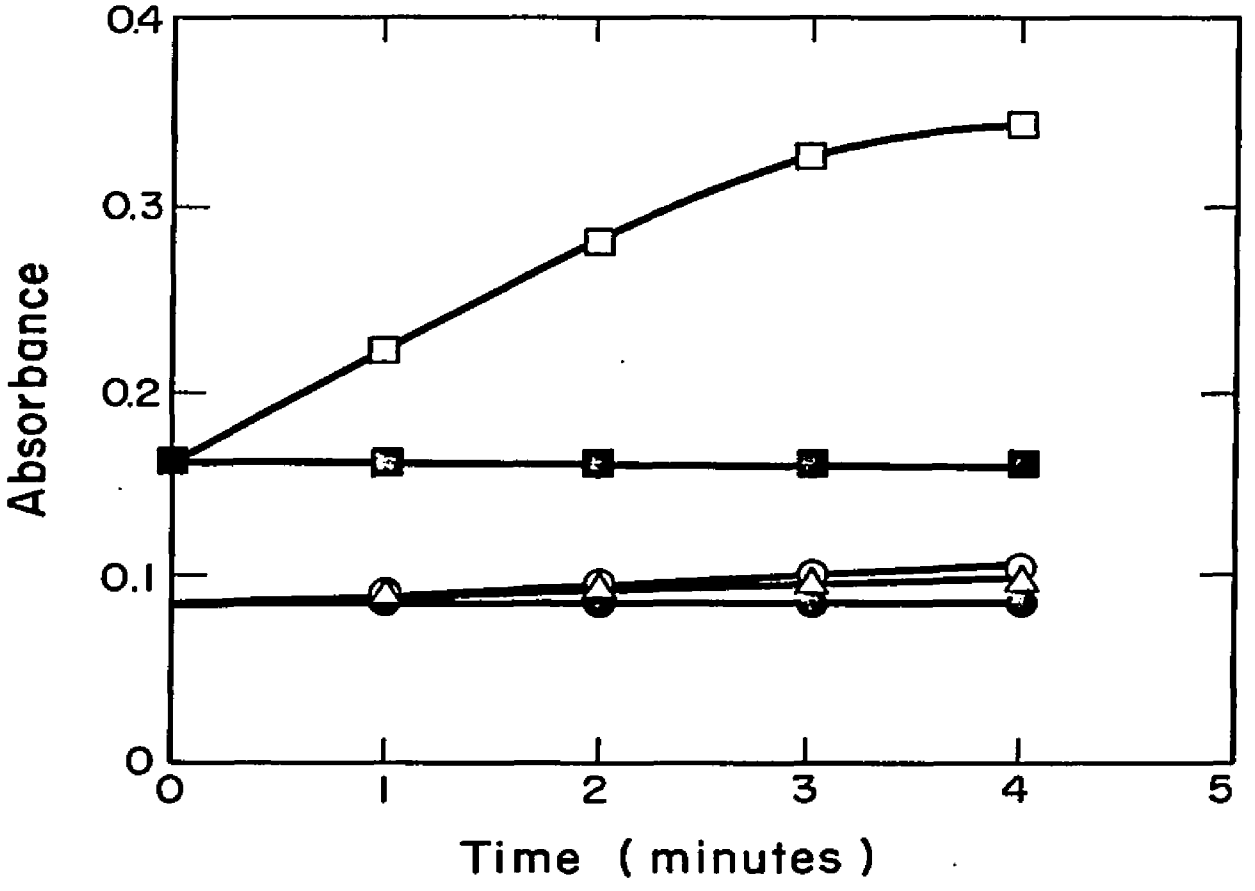
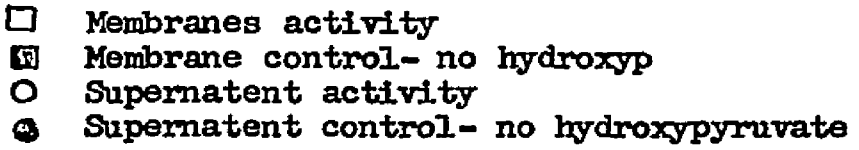

0.4
0.3
0.2
0.1
0
Absorbance
0
1
2
3
4
5
Time (minutes)

Figure 41. Graphs showing hydroxypyruvate reductase activity.



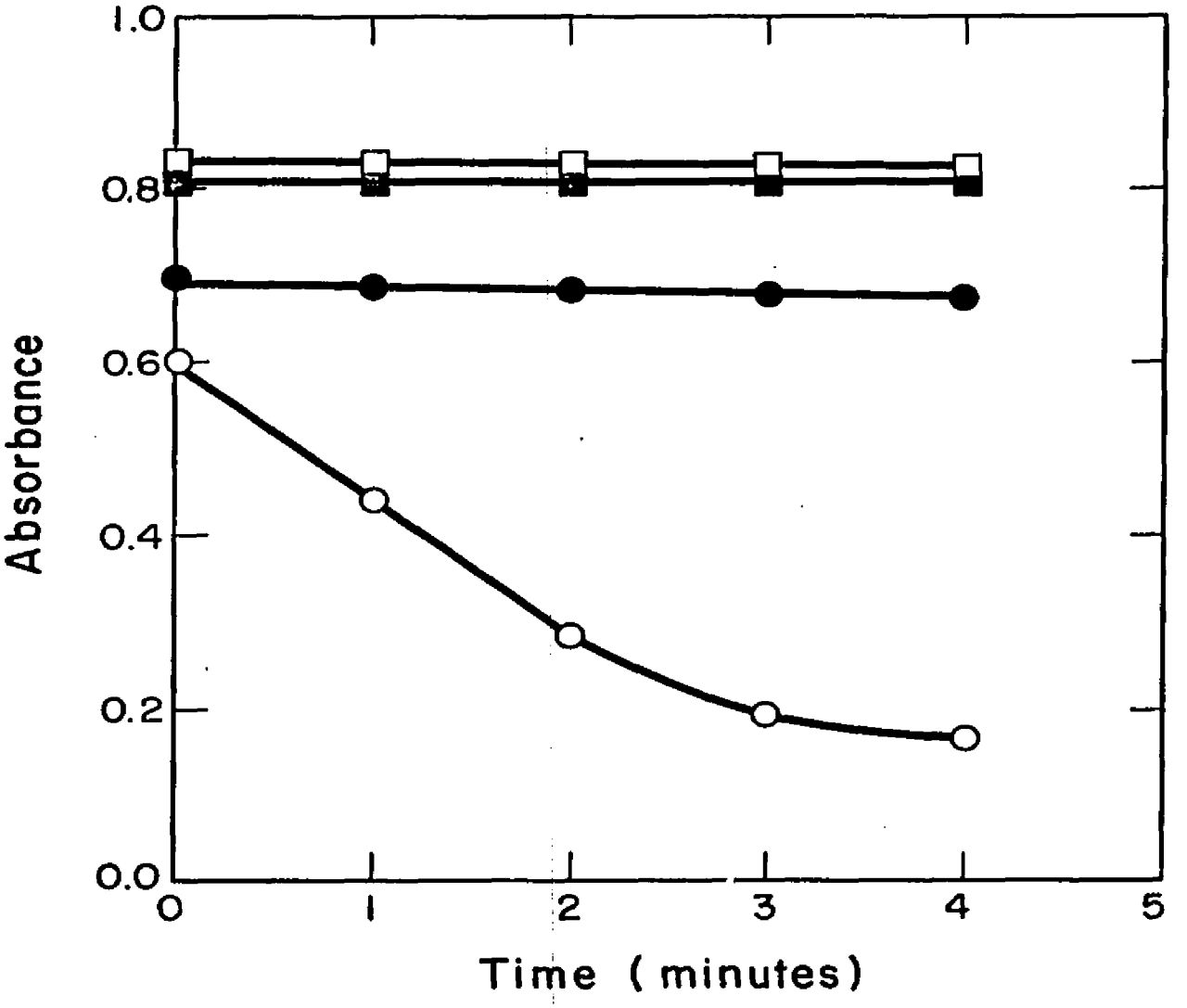

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
0
1
2
3
4
5
Time (minutes)

Figure 42. Graphs showing beta-hydroxybutyrate dehydrogenase activity.

□ membrane activity
■ membrane control- no beta-hydroxybutyrate
○ supernatent activity
● supernatent control-no beta-hydroxybutyrate

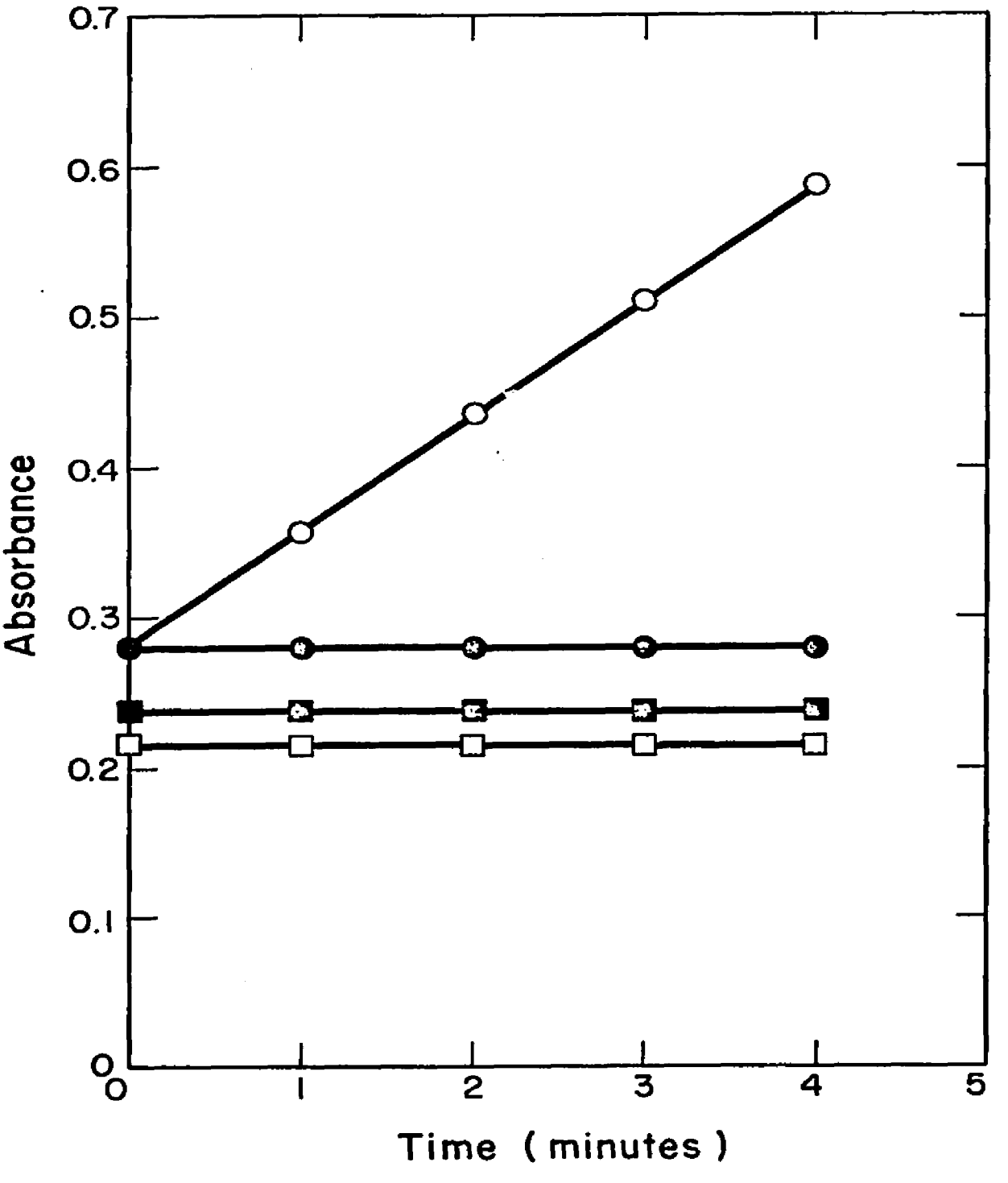

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
0
1
2
3
4
5
Time (minutes)

Table 20. Initial velocities and activities of enzymes in membrane and cytoplasmic preparations.

	Initial Velocity (nmoles/min)		Activity (nM/min./mg)	
	Cyt.	Mem.	Cyt.	Mem.
serine hydroxymethyltransferase	0.56	0	1.4	0
methanol dehydrogenase	303	0	78	0
formaldehyde dehydrogenase	3.4	0	0.87	0
formate dehydrogenase	51	0	13.1	0
NADH:cyt. C reductase	0	3.1	0	1.2
ATPase	3100	10600	910	2700
hydroxypyruvate reductase	21	0	5.38	0
beta-hydroxybutyrate dehydrogenase	17	0	5	0

The activities of the enzymes range from 0.87 to 2700 nanomoles/minute/mg protein. In this study, formaldehyde dehydrogenase had the lowest activity. This enzyme could function as a control point for the channeling of carbon into cell materials. The separation of activity between cytoplasm and membrane is very evident in these data. It is proposed that the extra-membrane enzymes are cytoplasmic rather than contained inside membrane vesicles. This is based upon the observation that many vesicles in the membrane fractions appear intact and yet most of the enzymes are restricted either to cytoplasm or membrane with no overlap of activity. If the enzymes were intravesicular, overlap of activity would be expected. The overlap of activity with ATPase has been observed before with other bacteria (Salton, 1971) and may be due to release of membrane bound enzyme , or to the fact that the ATPase is intravesicular. Since the intracytoplasmic membranes contain cytochromes, NADH-Cytochrome C reductase, and ATPase, they must serve as organelles of electron transport and energy entrapment. The oxidation and fixation of methane are cytoplasmic processes.

SUMMARY

1. Methylosinus trichosporium is an obligate methylotrophic Gram negative rod shaped bacterium.
2. A variety of amino acids, organic acids, pentoses, and hexoses enhance methane oxidation by this bacterium.
3. The bacterium has a generation time of about 5.3 hours.
4. The growth constant of this bacterium in log phase growth was about 0.1312 hours^{-1}.
5. The bacterium fixes about 50% of the methane oxidized into cell material.
6. One oxygen atom is consumed at each oxidative step in methane oxidation which is consistent with a monooxygenase.
7. High speed differential centrifugation fractions contain relatively purified intracytoplasmic membranes.
8. The intracytoplasmic membranes occur as flattened balloon like vesicles near the cell periphery.
9. The intracytoplasmic membranes are about 9 nanometers thick.
10. Vesicles formed by intracytoplasmic membranes are 16-300 nanometers in width.
11. The cell wall has an outer layer 6 nanometers thick and an inner layer 9.4 nanometers thick.
12. Fibers 10-70 nanometers in length attach the plasma membrane to the cell wall.

13. Intracytoplasmic membranes are passed along during budding.
14. The bacteria have intercellular connections, 30 X 75 nanometers, of unknown origin and unknown function.
15. 9.2% of cell dry weight and 27.9% of membrane dry weight were extracted as lipid.
16. Extracted cellular lipid consisted of 26.4% PHB, 39.1% simple lipids, and 34.5% phospholipids.
17. 83.4% of extracted membrane lipid was phospholipid.
18. Triglycerides were the main cellular simple lipid while free fatty acids were the principal simple lipid in membranes.
19. Phosphatidyl ethanolamine and phosphatidyl glycerol accounted for 94% of membrane phospholipid.
20. About 88% of the phospholipid fatty acids were 18:1, 12% of the fatty acids were 16:1.
21. Nonmembrane proteins average 33% polar amino acids.
22. Whole bacteria contained about 32% polar amino acids.
23. Membranes contained about 27% polar amino acids.
24. Intracytoplasmic membranes contained 5 major proteins ranging in molecular weight from 47,000 to 180,000.
25. The organism has a C_2 cytochrome with reduced absorption maxima at 416, 522, and 550 nanometers.
26. When oxidized, the C_2 Soret peak shifts to 410 nanometers.

27. This bacterium has an A cytochrome with absorption peaks at 445, 600, and 630 nanometers when reduced.
28. Methane is fixed into cell material by the cytoplasmic enzyme serine hydroxymethyl transferase.
29. An NAD independent methanol dehydrogenase is present in the cytoplasm.
30. The bacterium has a specific formaldehyde dehydrogenase in the cytoplasm.
31. Formate dehydrogenase, beta-hydroxybutyrate dehydrogenase, and hydroxypyruvate reductase are cytoplasmic enzymes.
32. NADH:cytochrome C reductase is a membrane enzyme.
33. The cytochromes are membrane associated.
34. ATPase was observed in both cytoplasm and membranes.
35. Intracytoplasmic membranes are organelles of electron transport and energy entrapment in this bacterium.
36. The intracytoplasmic membranes of M. trichosporium are morphologically and biochemically similar to the internal membranes of the photosynthetic and nitrifying bacteria.

APPENDIX

Figure 43. Standard curve for Methylosinus trichosporium showing concentration versus absorbance at 420 nm.

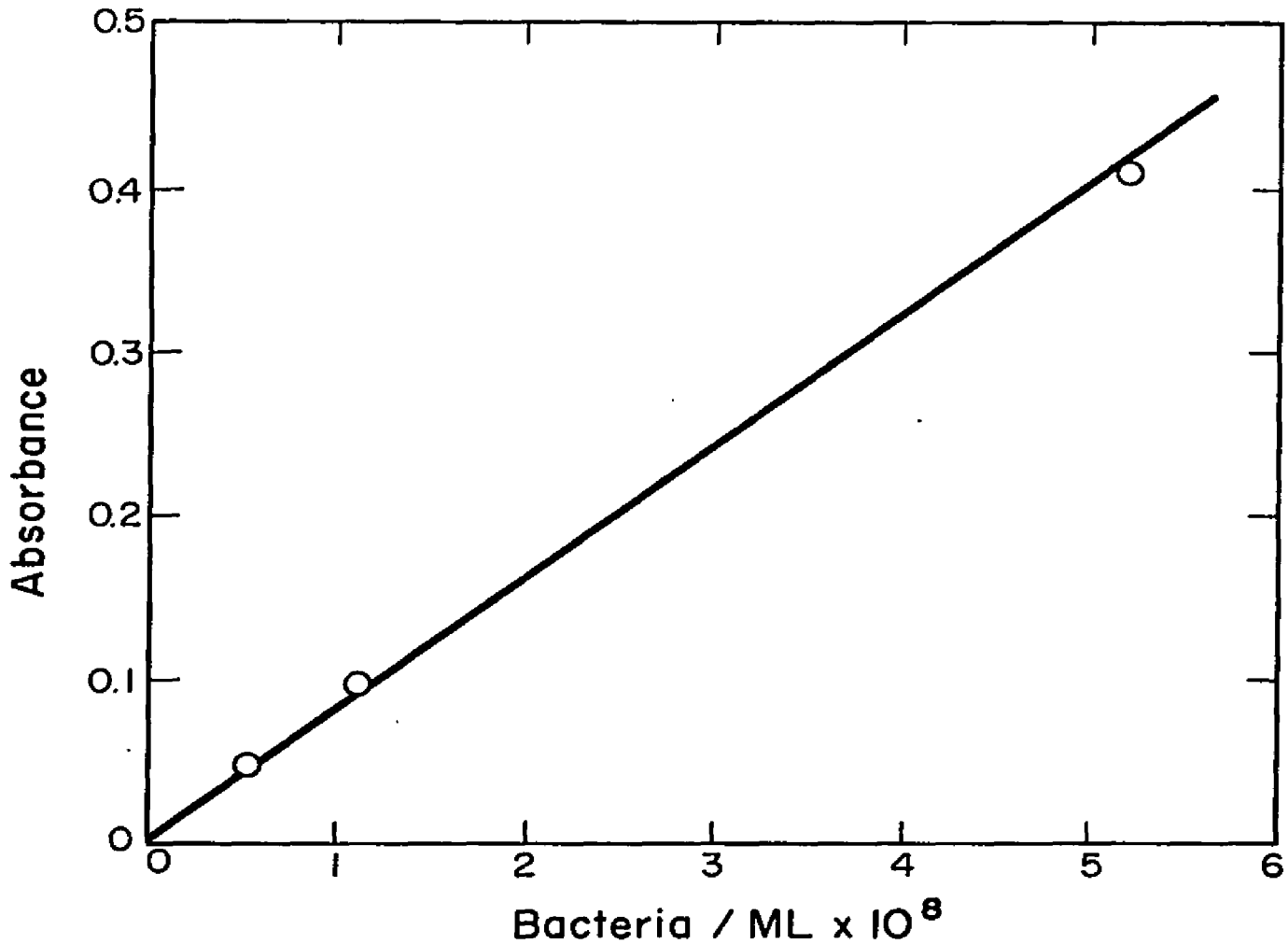

0.5
0.4
0.3
0.2
0.1
0
Absorbance
0
1
2
3
4
5
6
Bacteria / ML x 10⁸

Figure 44. Standard curve for protein(Lowry) showing concentration versus absorbance at 660 nm.

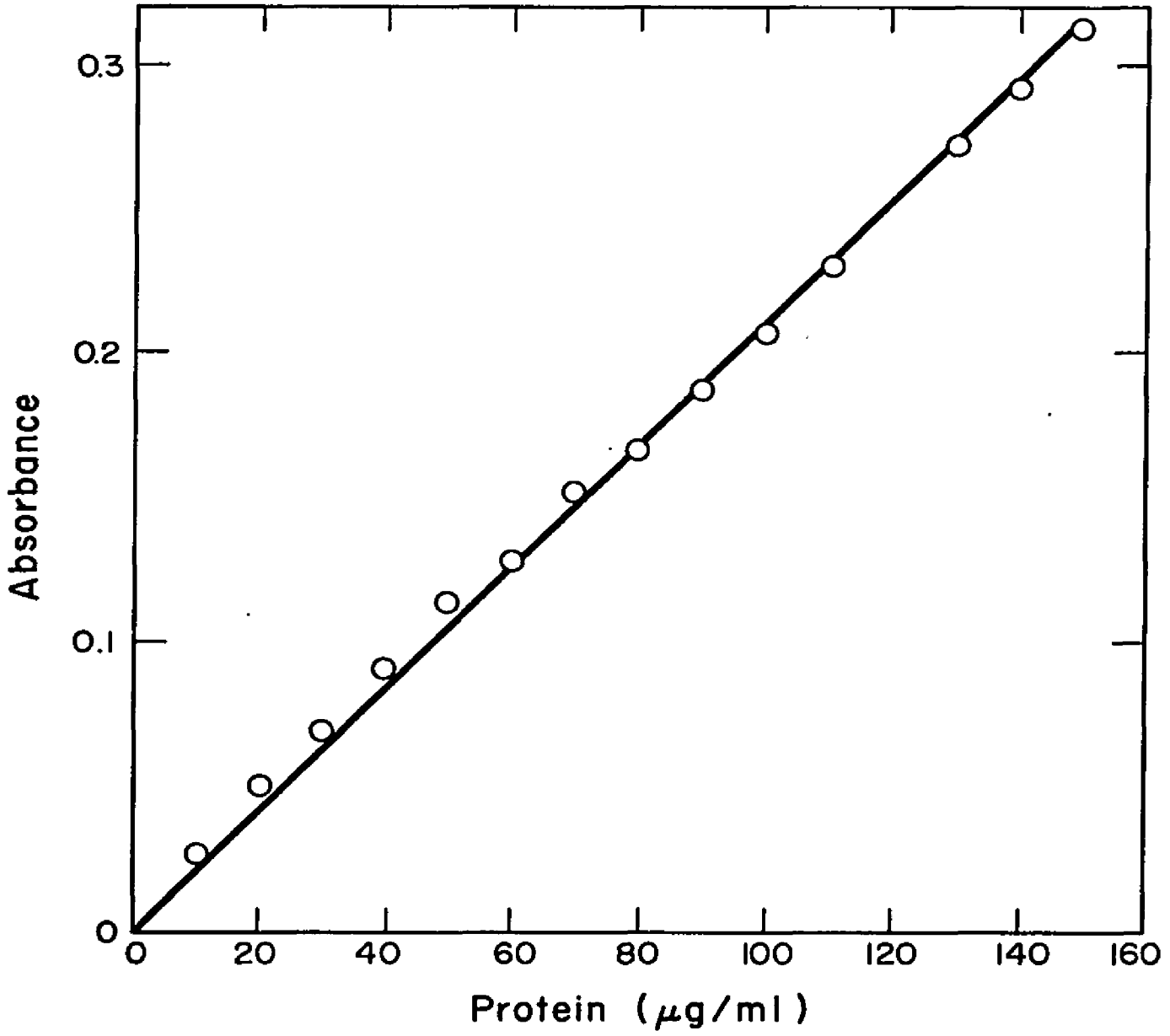

0.3
0.2
0.1
0
Absorbance
0
20
40
60
80
100
120
140
160
Protein (μg/ml)

Figure 45. Standard curve for glucose (anthrone) showing concentration versus absorbance at 620 nm.

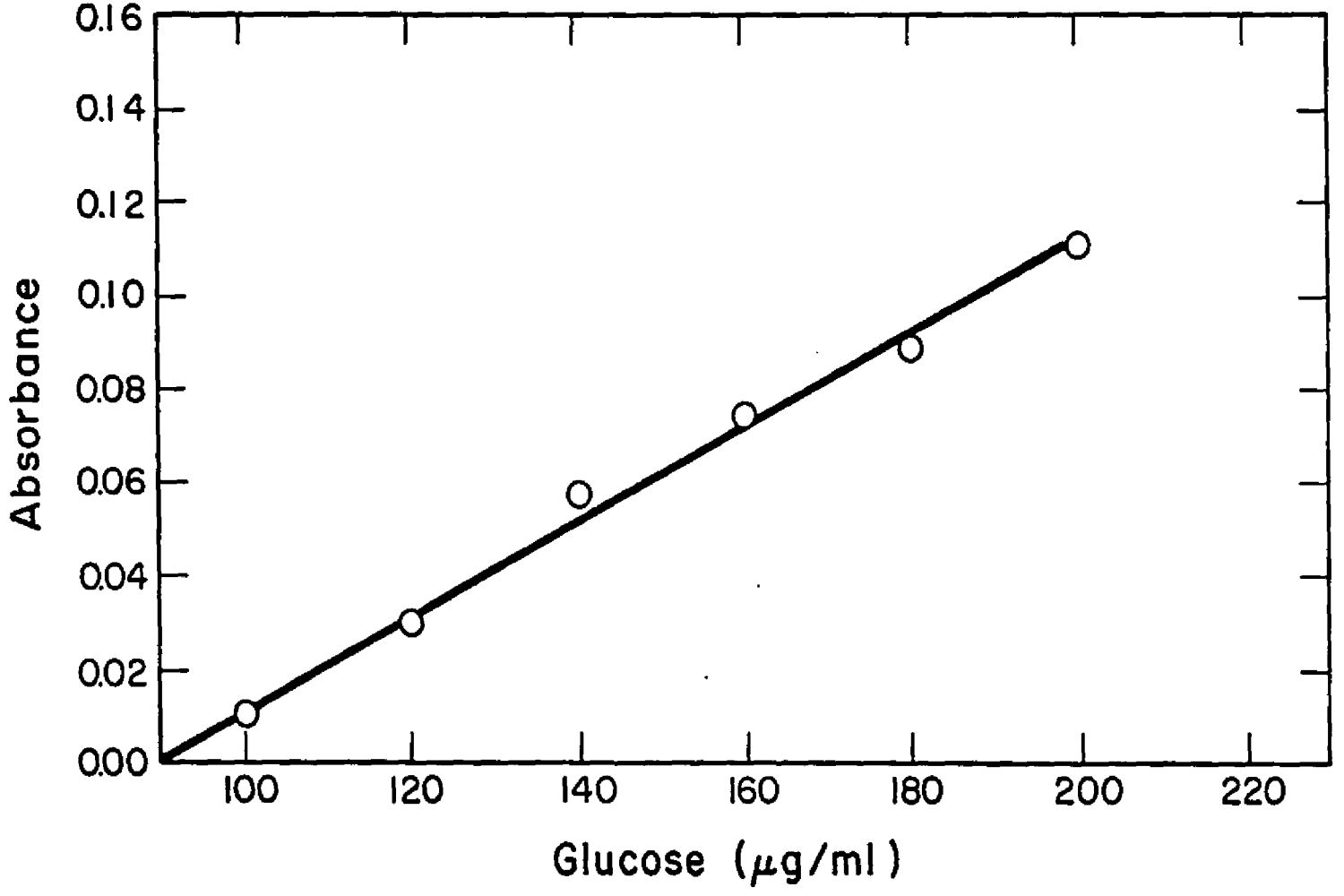

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance
100
120
140
160
180
200
220
Glucose (μg/ml)

Figure 46. Standard curve for formaldehyde (chromotropic acid) showing concentration versus absorbance at 570 nm.

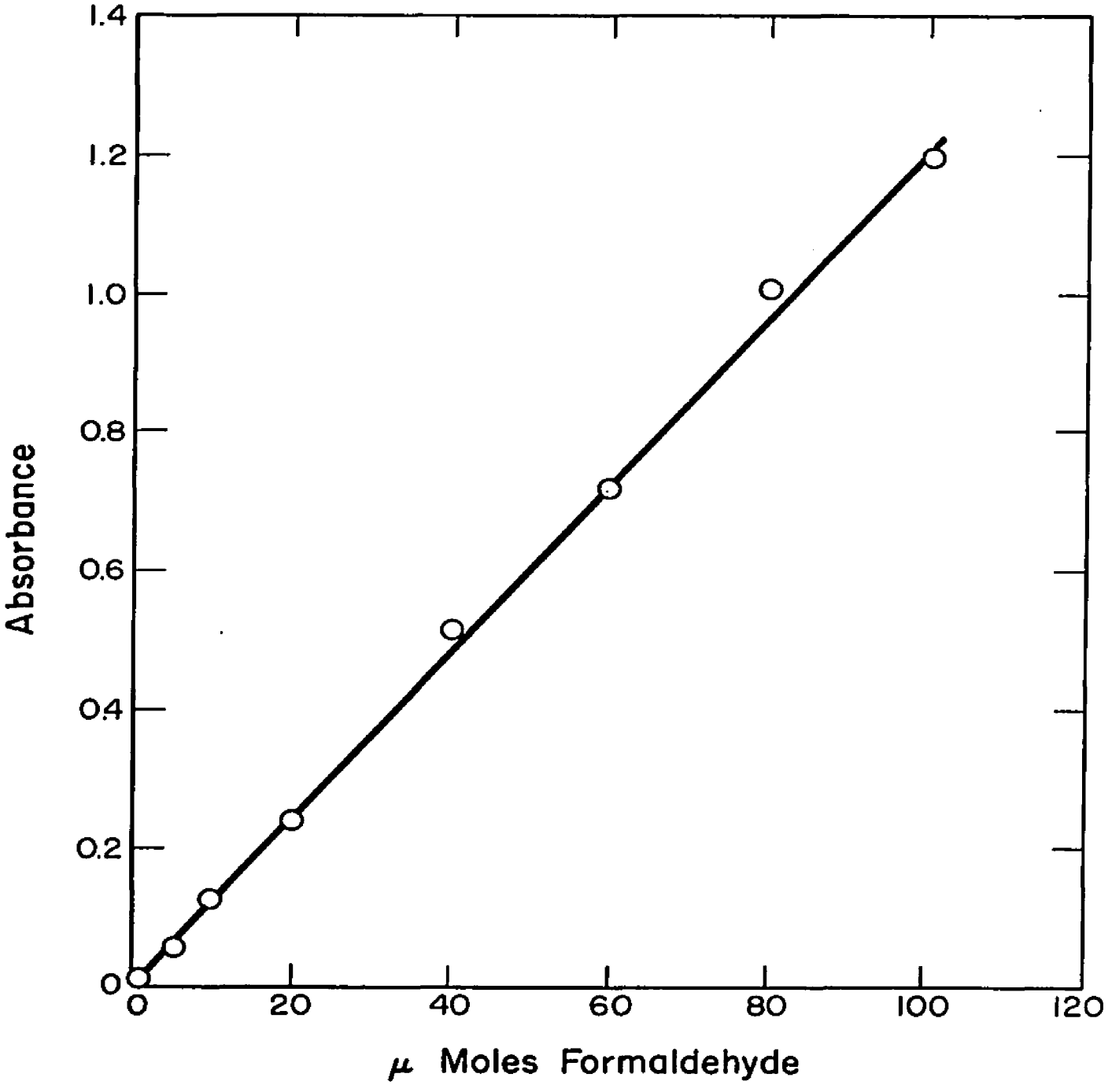

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Absorbance
0
20
40
60
80
100
120
μ Moles Formaldehyde

Figure 47. Standard curve for amino acids (ninhydrin) showing concentration versus absorbance at 570 nm.

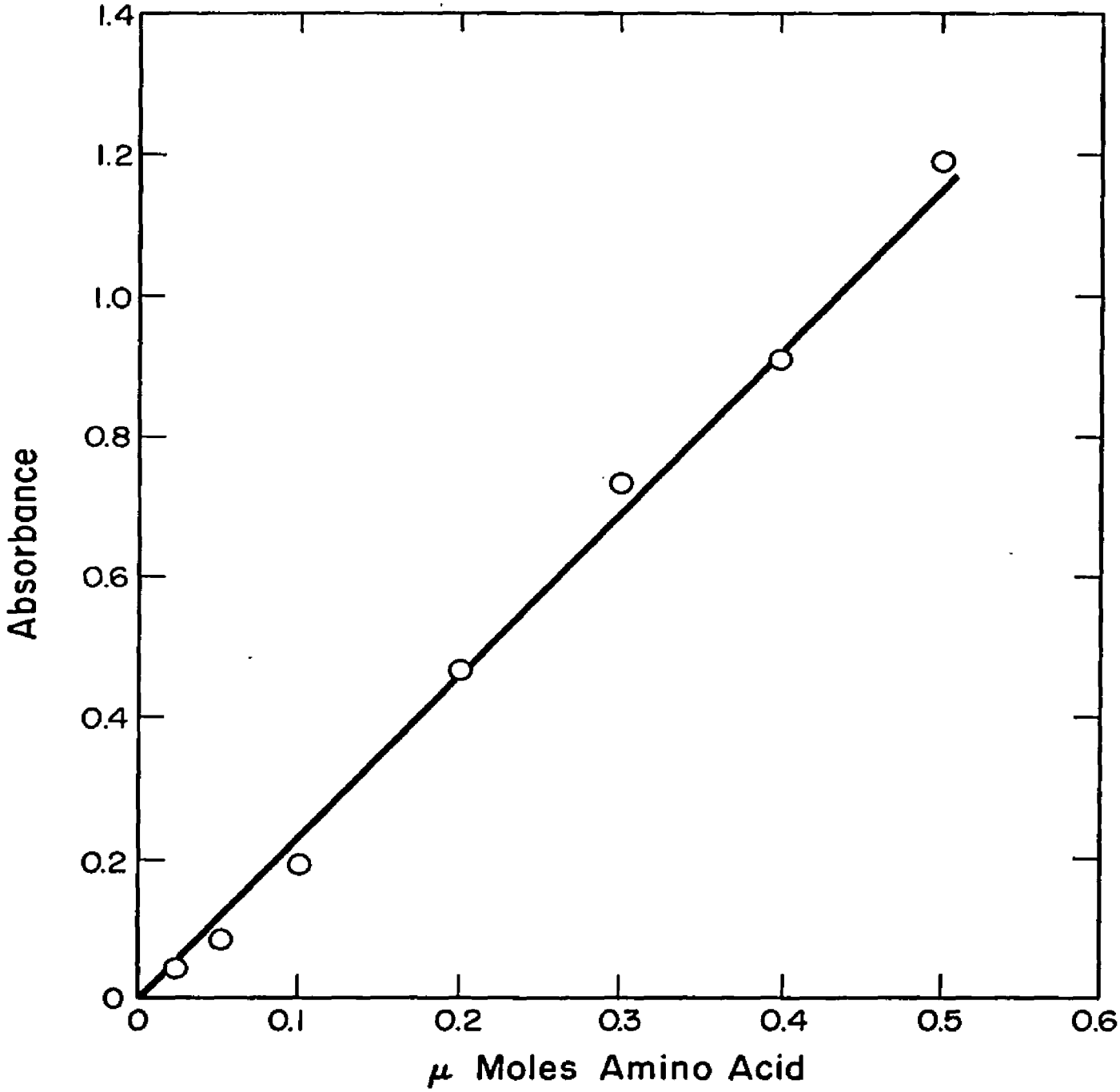

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Absorbance
0
0.1
0.2
0.3
0.4
0.5
0.6
μ Moles Amino Acid

Figure 48. Standard curve for inorganic phosphate (molybdate) showing concentration versus absorbance at 800 nm.

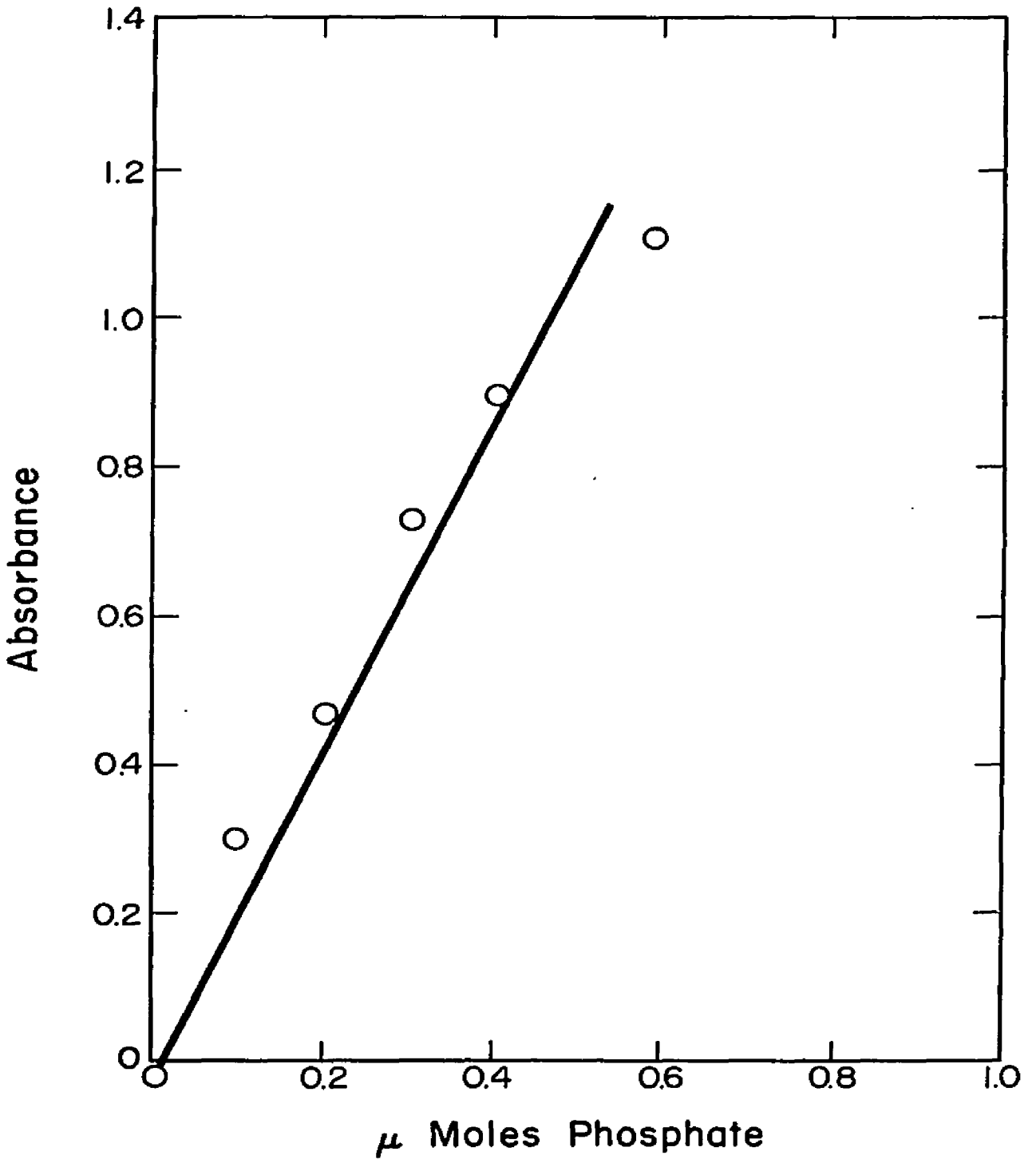
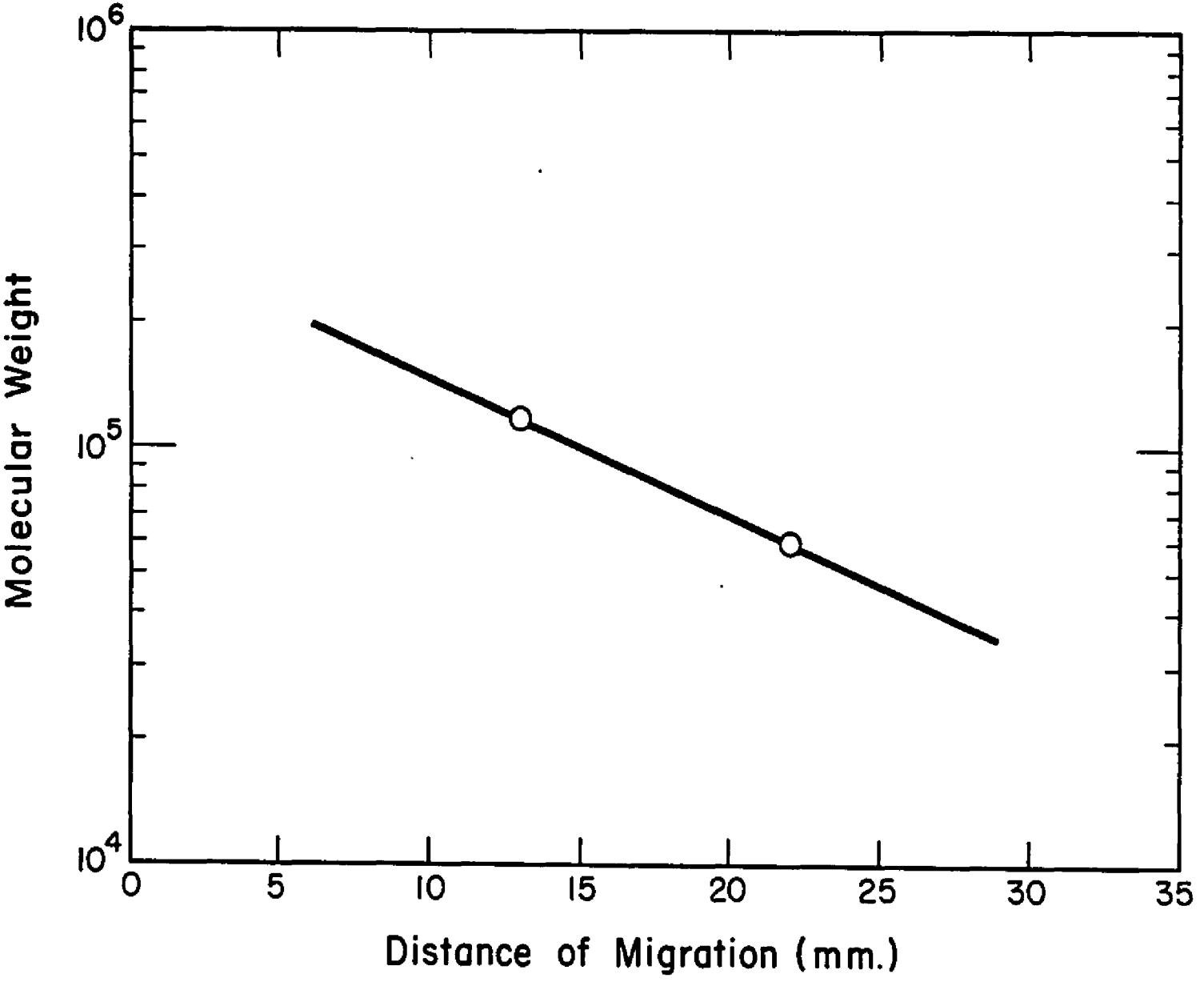

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Absorbance
0
0.2
0.4
0.6
0.8
1.0
μ Moles Phosphate

Figure 49. Graph relating protein migration distance in polyacrylamide gel to molecular weight based upon BSA standard.


Molecular Weight
10^6
10^5
10^4
0
5
10
15
20
25
30
35
Distance of Migration (mm.)

LITERATURE CITED

1. Ames, B.N. 1966. Assay of Inorganic Phosphate, Total Phosphate and Phosphatases. Methods in Enzymology. 8:115-118.

2. Blackwood, A.C. and E.P.P. Agnes. 1957. Identification of Beta-hydroxybutryric Acid in Bacterial Cells of Infrared Spectrophotometry. J. Bacteriol. 74:266-267.

3. Bligh, E.G. and W.J. Dyer. 1959. A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37:911-917.

4. Blumer, M., T. Chase, and S. W. Watson. 1969. Fatty Acids in the Lipids of Marine and Terrestrial Nitrifying Bacteria. J. Bacteriol. 99:366-370.

5. Brown, L.R. and R.J. Strawinski. 1958. Intermediates in the Oxidation of Methane. Bacteriol. Proc. p. 122.

6. Brown, L.R., R.J. Strawinski, and C.S. McCleskey. 1964. The Isolation and Characterization of Methanomonas methanoxidans. Can. J. Microbiol. 10:791-799.

7. Coleman, R. 1973. Membrane Bound Enzymes and Membrane Ultrastructure. Biochim. Biophys. Acta. 300:1-30.

8. Danielli, J.F. and H.A. Davson. 1936. A Contribution to the Theory of Permeability of Thin Films. J. Cell. Comp. Physiol. 9:89-92.

9. Davies, S.L. and R. Whittenbury. 1970. Fine Structure of Methane and Other Hydrocarbon Utilizing Bacteria. J. Gen. Microbiol. 61:227-233.

10. Davies, T.R. 1973. Isolation of Bacteria Capable of Utilizing Methane as a Hydrogen Donor in the Process of Denitrification. Water Res. 7:575-579.

11. Davis, B.D., R. Dulbecco, H.N. Eisen, and H.S. Ginsberg. W.B. Wood-Microbiology. Harper and Row

12. Davis, J.B., V.F. Coty, and J.P. Stanley. 1964. Atmospheric Nitrogen Fixation by Methane Oxidizing Bacteria. J. Bacteriol. 88:468-472.

13. D'Mello, J. 1972. A Study of the Amino Acid Composition of Methane Utilizing Bacteria. J. Appl. Bacteriol. 1:145-148.

14. Dworkin, M. and J.W. Foster. 1956. Studies on Pseudomonas methanica (Söhngen). J. Bacteriol. 72:646-659.

15. Eller, D. and D.G. Lundgren. 1968. Morphology of Poly-beta-hydroxybutyrate Granules. J. Mol. Biol. 35:489-502.

16. Emerson, R.W. 1870. Society and Solitude. Fields, Osgood, and Co., Boston, Mass. p. 142.

17. Folch, J., M. Lees, and G.H. Sloane Stanley. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem. 226:497.

18. Folin, O. and V. Ciocalteu. 1927. On Tyrosine and Tryptophane Determinations in Protein. J. Biol. Chem. 73:627-650.

19. Foster, J.W. and R.H. Davis. 1966. A Methane Dependent Coccus with Notes on Classification and Nomenclature of Obligate Methane Utilizing Bacteria. J. Bacteriol. 91:1924-1931.

20. Giglioni, I. and G. Masoni. 1914. Nuove osservazioni sull'assorbimento Biologicao del Metano; e sulla Distribuzione nei Terreni, nelle Melme e negli ingrassi, degli Organismi Metanici di Kaserer e di Söhngen. Pisa Universita, Instituto di Chimica Agraria, Studi e Ricerche. 22:76-94.

21. Gorter, E. and R. Grendel. 1925. On Bimolecular Layers of Lipoid on the Chromocytes of Blood. J. Exp. Med. 41:439-443.

22. Goswami, S.K. and C.F. Frey. 1971. Spray Detection of Phospholipids on Thin Layer Chromatograms. J. Lipid Res. 12:509-510.

23. Guidotti, G. 1972a. The Composition of Biological Membranes. Arch. Int. Med. 129:194-201.

24. Guidotti, G. 1972b. Membrane Proteins. Ann. N.Y. Acad. Sci. 195:139-141.

25. Hagen, P.O. 1966. Phospholipids of Bacteria with Extensive Intracytoplasmic Membranes. Science. 151:1546.

26. Häggstrom, L. 1969. Studies on Methanol Utilizing Bacteria. Biotech. Bioeng. 11:1043-1054.

27. Hamer, G. and C.G. Heden. 1967. Methane as a Carbon Substrate for the Production of Microbiol Cells. Biotech. Bioeng. 9:499-514.

28. Hanes, C.S. and F.A. Isherwood. 1949. Separation of Phosphoric Esters on the Filter Paper Chromatogram. Nature. 164:1107-1112.

29. Harder, W. and J.R. Quayle. 1971a. The Biosynthesis of Serine and Glycine in Pseudomonas AM1 with Special Reference to Growth on Carbon Sources other than C_1 Compounds. Biochem. J. 121:753-762.

30. Harder, W. and J.R. Quayle. 1971b. Aspects of Glycine and Serine Biosynthesis During Growth of Pseudomonas AM1 on C_1 Compounds. Biochem. J. 121:763-769.

31. Harrington, A.A., and R.E. Kallio. 1960. Oxidation of Methanol and Formaldehyde by Pseudomonas methanica. Can. J. Microbiol. 6:1-7.

32. Hatefi, Y., M.J. Osborn, L.D. Kay, and F.M. Huennekens. 1957. Hydroxymethyl Tetralydrofolic Dehydrogenase. J. Biol. Chem. 227:637-647.

33. Hazeu, W. and P.J. Steenis. 1970. Isolation and Characterization of Two Vibrio-Shaped Methane Oxidizing Bacteria. Ant. van Leeuwenhoek. 36:67-72.

34. Heptinstall, J. and J.R. Quayle. 1970. Pathways Leading to and from Servine During Growth of Pseudomonas AM1 on C_1 Compounds or Succinate. Biochem. J. 117:563-572.

35. Higgins, I.J. and J.R. Quayle. 1970. Oxygenation of Methane by Methane Grown Pseudomonas methanica and Methanomonas methanoxidans. Biochem. J. 118:201-208.

36. Hutton, W.E. and C.E. Zobell. 1949. The Occurance and Characteristics of Methane Oxidizing Bacteria in Marine Sediments. J. Bacteriol. 58:463-473.

37. Hutton, W.E. and C.E. Zobell. 1952. Production of Nitrite from Ammonia by Methane Oxidizing Bacteria. J. Bacteriol. 65:216-219.

38. Johnson, J.L. and K.L. Temple. 1962. Some Aspects of Methane Oxidation. J. Bacteriol. 84:456-458.

39. Johnson, P.A. and J.R. Quayle. 1965. Microbial Metabolism of C_1 Compounds. Biochem. J. 95:859.

40. Robertson, J.D. 1972. The Structure of Biological Membranes. Arch. Int. Med. 129:202-228.

41. Kaneda, T. and J.M. Roxburgh. 1959. Servine as an Intermediate in the Assimilation of Methanol by a Pseudomonas. Biochem. Biophys. Acta. 33:106-110.

42. Kaserer, H. 1905. Ueber die Oxydation des wasserstofes und des Methane durch Mikroorganismen. Z. Landv. Versuchsw. Deut. Oesterr. 8:789.

43. Kemp, M.B. and J.R. Quayle. 1967. Microbial Growth or C_1 Compounds: Uptake of C^{14} Formaldehyde and C^{14} Formate by Methane Grown Pseudomonas methanica and Determination of the Hexose Labelling Pattern After Brief Incubation with C^{14} Methanol. Biochem. J. 102:94-102.

44. Large, P.J., D. Peel, and J.R. Quayle. 1961. Microbial Growth on C_1 Compounds: Synthesis of Cell Constituents by Methanol and Formate Grown Pseudomonas AM1. Biochem. J. 81:470-480.

45. Large, P.J., D. Peel, and J.R. Quayle. 1962. Microbial Growth on C_1 Compounds: Distribution of Radioactivity in Metabolites of Methanol Grown Pseudomonas AM1 After Incubation with C^{14} Methanol and C^{14} Bicarbonate. Biochem. J. 82:483-488.

46. Large, P.J. and J.R. Quayle. 1963. Microbial Growth on C_1 Compounds: Enzyme Activities in Extracts of Pseudomonas AM1. Biochem. J. 87:386-396.

47. Law, J.H. and R.S. Slepecky. 1961. Assay of Poly-beta-hydroxybutric Acid. J. Bacteriol. 82:33-36.

48. Lawrence, A.J., M. B. Kemp, and J.R. Quayle. 1970. Synthesis of Cell Constituents by Methane Grown Methylococcus capsulatus and Methanomonas methanoxidans. Biochem. J. 116:631-639.

49. Lawrence, A.J. and J.R. Quayle. 1970. Alternative Carbon Assimilation Pathways in Methane Utilizing Bacteria. J. Gen. Microbial. 63:371-374.

50. Leadbetter, E.R. and J.W. Foster. 1958. Studies on Some Methane Utilizing Bacteria. Arch. for Mikrobiol. 30:91-118.

51. Leadbetter, E.R. and J.W. Foster. 1959. Incorporation of Molecular Oxygen in Bacterial Cells Utilizing Hydrocarbons for Growth. Nature. 184:1428-1429.

52. Leadbetter, E.R. and J.W. Foster. 1960. Bacterial Oxidation of Gaseous Alkanes. Arch. for Mikrobiol. 35:92-104.

53. Lenard, J. and S.J. Singer. 1966. Protein Conformation in Cell Membrane Preparations as Studied by Optical Rotatory Dispersion and Circular Dichroism. Prac. Nat. Acad. Sci. U.S.A. 56:1828-1835.

54. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein Measurement With the Folin Phenol Reagent. J. Biol Chem. 193:265-275.

55. Mahler, H. and E. Cordes. 1966. Biological Chemistry. Harper and Row, New York, N.Y.

56. Nageli, C. and C. Cramer. 1972. Pflanzephysiologische Untersuchungen (1855). The Development of Ideas on Membrane Structure. Sub. Cell. Biochem. 1:363-373.

57. Naguib, M. and J. Overbeck. 1970. On Methane Oxidizing Bacteria in Fresh Waters. Zeit. für Allg. Mikrobiol. 10:17-36.

58. Nechaeva, N.B. 1949. Two Species of Methane Oxidizing Mycobacteria. Mikrobiologiya. 18:310-317.

59. Oelze, J. and G. Drews. 1972. Membranes of Photosynthetic Bacteria. Biochem. Biophys. Acta. 265:209-239.

60. O'Leary, W.M. 1967. The Chemistry and Metabolism of Microbial Lipids. The World Pub. Co., Cleveland, Ohio.

61. Op Den Kamp, J.A.F. and L.L.M. Van Deenen. 1969. Bacterial Phospholipids and Membranes as in Structural Aspects of Lipoproteins in Living Systems. Academic Press. New York, N.Y. pp. 227-325.

62. Overton, E. 1895. Uber die osmotischen Eigenshafter der Lebenden Pflanzen und tierzelle. Vjschr. Naturf. Ges Zurich. 40:159-201.

63. Patel, R.N., H.R. Bose, W.J. Mandy, and D.S. Hoare. 1972. Physiological Studies of Methane and Methanol Oxidizing Bacteria: Comparison of a Primary Alcohol Dehydrogenase from Methylococcus capulatus (Texas Strain) and Pseudomonas Species M27. J. Bacteriol. 110:570-577.

64. Patel, R.N. and D.S. Hoare. 1971. Physiological Studies of Methane and Methanol Oxidizing Bacteria: Oxidation of C-1 Compounds by Methylococcus capsulatus. J. Bacteriol. 107:187-192.

65. Peel, D. and J.R.Quayle. 1961. Microbial Growth on C-1 Compounds: Isolation and Characterization of Pseudomonas AM1. Biochem. J. 81:465-469.

66. Perry, J.J. 1968. Substrate Specificity in Hydrocarbon Utilizing Microorganisms. Ant. van Leeuwenhoek. 34:27-36.

67. Proctor, H.M., J.R. Norris, and D.W. Ribbons. 1969. Fine Structure of Methane-Utilizing Bacteria. J. Appl. Bacteriol. 32:118-121.

68. Reynolds, E.S. 1963. The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell. Biol. 17:208.

69. Ribbons, D.W., J.E. Harrison, and A.M. Wadzinski. 1970. Metabolism of Single Carbon Compounds. Ann. Rev. Microbiol. 24:135-158.

70. Ribbons, D.W. and I.J. Higgens. 1971. Enzymic Oxidation of Methane by Particles from Methylococcus capulatus. Bacteriol. Proc. p. 107.

71. Ribbons, D.W. and J.L. Michalover. 1970. Methane Oxidation by Cell-Free Extracts of Methylococcus capulatus. FEBS Letters 11:41-44.

72. Rosen H. 1957. A Modified Ninhydrin Colorimetric Analysis for Amino Acids. Arch. Biochem. Biophys. 67:10-15.

73. Saber, H.A., R.A. Harte, and E.K. Saber. 1970. Handbook of Biochemistry. Chem.Rubber Co. Cleveland, Ohio. pp. C:282-287.

74. Salton, M. R. 1971 Bacterial Membranes. Crit. Rev. Microbiol. May:161-197.

75. Sheehan, B.T. and M.J. Johnson. 1971. Production of Bacterial Cells from Methane. Appl. Microbiol. 21:511-515.

76. Singer, S.J. 1973. Are Cell Membranes Fluid? Science. 180:983.

77. Singer, S.J. and G.L. Nicolson. 1972. The Fluid Mosaic Model of Membrane Structure. Nature. 175:720.

78. Sjöstrand, F.S. 1969. Morphological Aspects of Lipoprotein Structures, in Structoral and Functional Aspects of Lipoprotein in Living Systems. Academic Press. New York, N.Y. pp. 73-139.

79. Skipshi, V.P. and M. Barclay. 1969. Thin Layer Chromatography of Lipids. Methods in Enzymology. 14:530-598.

80. Smith, U. and D.W. Ribbons. 1970. Fine Structure of Methanomonas methanoxidans. Arch. Mikrobiol. 74:116-122.

81. Smith, U., D.W. Ribbons, and D.S. Smith. 1970. The Fine Structure of Methylococcus capsulatus. Tissue and Cell. 2:513-520.

82. Söhngen, N.L. 1906. Ueber Bakterien, welch Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. Zentr. Bakt. Parasitenk. 15:513-517.

83. Stocks, P.K. and C.S. McCelskey. 1964. Morphology and Physiology of Methanomonas methanoxidans. J. Bacteriol. 88:1071-1077.

84. Strawinski, R.J. and L.R. Brown. 1957. Isolation Criteria and Characterization of a New Methane Oxidizing Bacterium. Bacteriol. Proc. p. 18.

85. Strawinski, R.J. and J.A. Tortovich. 1955. Preliminary Studies of Methane Oxidizing Bacteria and Their Possible Role in Oil Prospecting. Bacteriol. Proc. p. 27.

86. Vanderkooi, G. and R.A. Capaldi. 1972. A Comparative Study of the Amino Acid Compositions of Membrane Proteins and Other Proteins. Ann. N.Y. Acad. Sci. 195:135-138.

87. Vary, P.S. and M.J. Johnson. 1967. Cell Yields of Bacteria Grown on Methane. Appl. Microbiol. 15:1473-1478.

88. Watson, S.W. 1965. Characteristics of a Marine Nitrifying Bacterium, Nitrocystis oceanus sp. n. Limnol and Ocean. 10:274-289.

89. Weaver, T.L. and P.R. Dugan. 1972. The Eutrophication Implications of Interactions between Naturally Occurring Particulates and Methane Oxidizing Bacteria. Water Res. 6:817-828.

90. Wertlieb, D. and W. Vishniac. 1967. Methane Utilization by a Strain of Rhodopseudomonas gelatinosa. J. Bacteriol. 93:1722-1724.

91. White, D.C. 1968. Lipid Composition of the Electron Transport Membrane of Haemophilis parainfluenzoe. J. Bacteriol. 96:1159-1170.

92. Whittenbury, R. 1969. Microbiol Utilization of Methane. Proc. Biochem. 1:51-56.

93. Whittenbury, R., S.L. Davies, and J.F. Davey. 1970. Exospores and Cysts formed by Methane Utilizing Bacteria. J. Gen. Microbiol. 61:219-231.

94. Whittenbury, R., K.C. Phillips, and J.F. Wilkinson. 1970. Enrichment, Isolation, and Some Properties of Methane Utilizing Bacteria. J. Gen. Microbiol. 61:205-218.

95. Wolnak, B., B.H. Andreen, J.A. Chisholm, and M. Saadek. 1967. Fermentation of Methane. Biotech. Bioeng. 9:57-76.