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Marine Microbiology

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Five-Year Progress Report

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I. Abstract

The Atomic Energy Commission has supported our investigations on marine microbiology for the past five years. During this period we studied the role of bacteria in the nitrogen cycle, specifically concentrating on the organisms responsible for microbiological oxidation of ammonia to nitrite and nitrite to nitrate. The distribution, rates of in situ reactions, fine structure and biochemical properties of these organisms were detailed. Rates of urea, acetate, and glucose decomposition in both inshore and offshore waters were determined using labelled compounds and the significance of these degradations in the hydrosphere was examined. Also a new test for the detection of free bacterial lipopolysaccharides, using a lysate from the "blood" of Limulus polyphemus, was explored and work has been initiated to use this test to quantitate the bacterial biomass in seawater.

II. Introduction

In this progress report we will summarize the investigations on marine microbiology supported by the Atomic Energy Commission for the past five years.

Marine bacteria mineralize organic matter and recycle plant nutrients in oceanic environments. The rate of biological productivity in the oceans is controlled, to a large extent, by the rate at which bacteria recycle plant nutrients. Thus a detailed knowledge of marine bacteria is necessary in order to understand the factors controlling the biological cycle in the ocean.

Our studies were concerned primarily with the role of bacteria in the nitrogen cycle and the breakdown of organic substrates in the marine environment. During the last five years, a major fraction of time was spent studying the process of nitrification (i.e., the oxidation of ammonia to nitrite and nitrite to nitrate) in the oceans of the world, and this review deals primarily with accomplishments in this area.

Nitrification is carried out by two groups of chemoautotrophic bacteria, the first group oxidizes ammonia to nitrite and the second group oxidizes nitrite to nitrate. These bacteria derive energy from these inorganic oxidations and satisfy their carbon needs by carbon dioxide fixation. Since nitrate is one of the most important plant nutrients in the ocean, nitrification in the marine environment merits considerable study. In the deep waters of the ocean, nitrate comprises over 70 percent of the combined nitrogen (Vaccaro, 1962). Since ammonia

cannot be spontaneously oxidized to nitrate in the ocean, it had been assumed that nitrifying bacteria were primarily responsible for the production of nitrate in the ocean.

Nitrifying bacteria were originally discovered in soils in 1892 (Winogradsky) and shortly thereafter, numerous investigators attempted to isolate them from the oceans. One worker (Thomsen, 1908) cultured these bacteria from seawater collected within a mile of shore but failed to culture them from offshore waters. Thus for many years it was thought oceanic nitrifying bacteria did not exist or were confined to inshore waters. It was not until our investigations, supported by the Atomic Energy Commission, that the first marine nitrifying bacterium was isolated from open ocean waters. This bacterium, an ammonia oxidizer, was a new species and was named Nitrosocystis oceanus (Watson, 1965). This organism has been recently renamed Nitrosococcus oceanus (Watson, 1971b). Since this original discovery, we have isolated several hundred strains of marine ammonia and nitrite oxidizers; examined their distribution; rates of nitrification; detailed the types of organisms involved and studied their fine structure and biochemistry.

III. Genera and Species of Nitrifying Bacteria

Prior to this study, five genera of ammonia oxidizers and two genera of nitrite oxidizers had been described. All of these genera were discovered either by Helene or Serge Winogradsky during the period from 1892 to 1935 (S. Winogradsky, 1892; 1931; S. Winogradsky, H. Winogradsky, 1933; H. Winogradsky, 1935a; 1935b). None of the original strains were

maintained in laboratory culture. The descriptions of these organisms were so brief that it was often impossible to identify an organism from the original description. Later investigations found that all ammonia oxidizers cultured belonged to the genus Nitrosomonas and the nitrite oxidizers to the genus Nitrobacter. The inability of workers to find other nitrifying genera led to the generally accepted hypothesis that only two valid genera existed. In the present study we established that there are at least four genera of ammonia oxidizers and four genera of nitrite oxidizers and we have revised the taxonomy of this group (Watson 1971b, see enclosed reprint).

A constant flow of microorganisms occurs between the terrestrial and marine environments. We wished to establish whether nitrifying bacteria moved between the two environments and if so, whether they were similar or identical. We cultured nitrifying bacteria from both environments and described their morphology and growth requirements in an effort to establish if there was an exchange of these bacteria between the two environments.

The first organism, Nitrosococcus oceanus (Watson, 1965) (Fig. 1), isolated from the open ocean was a large spherical organism with cytomembranes which formed flattened lamellae in the internal regions of the cells. This bacterium was not found in the terrestrial environment and could not survive in a medium consisting of less than 50 percent seawater. All present evidence suggests that this bacterium lives only in oceanic environments.

Nitrosococcus oceanus, while cultured repeatedly from both the Atlantic and Pacific Oceans, is not the predominant ammonia oxidizer in the oceans. The most common oceanic ammonia oxidizer is rod shaped and belongs to the genus Nitrosomonas. This organism, like the terrestrial species of Nitrosomonas, has cytomembranes arranged as flattened lamellae in the peripheral regions of the cell (Fig. 2). While the shape and the internal morphology of the marine species is similar or identical to the terrestrial species, the two forms were easily distinguished from each other. The marine species had an additional cell wall layer lying external to the double track lipopolysaccharide layer. This additional cell wall layer was composed of repeating subunits arranged in a molecular array. Two different arrangements of these subunits were found (Fig. 3 and 4). We examined several hundred terrestrial and marine strains of Nitrosomonas and these subunits were always present in the marine strains and absent in the terrestrial strains.

Terrestrial strains of Nitrosomonas appeared to be rapidly killed when they entered the marine environment. Like N. oceanus, marine strains of Nitrosomonas would not grow in media composed of less than 50 percent seawater.

While only two genera of marine ammonia oxidizers were found, three genera of terrestrial species were isolated and studied. Nitrosomonas species were present in most soil samples but were not always the predominant nitrifiers. One of the other ammonia oxidizers commonly present

was Nitrosospira briensis (Fig. 5) (Watson, 1971a). This bacterium had not been cultured since its original discovery (S. Winogradsky and H. Winogradsky, 1933). In our studies this ammonia oxidizer was isolated from the soils of Crete, the Greek mainland, Switzerland and from Cape Cod in Massachusetts. In soil it frequently outnumbered cells of Nitrosomonas species.

Nitrosospira briensis cells formed tightly coiled spirals (Fig. 5) and lacked any type of cytomembrane system usually present in other ammonia oxidizers (Fig. 6). These cells did not grow in seawater and to date have not been isolated from the marine environment.

Also discovered during this investigation was a fourth genus of an ammonia oxidizing bacterium. This organism was lobular in shape and had the cytoplasm partially compartmentalized by cytomembranes (Fig. 7) (Watson et al., 1971). This species was called Nitrosolobus multiformis. This ubiquitous bacterium was isolated from the following locations: Paramaribo, Surinam; Lauanda, Angola; Windhoek, Southwest Africa; Yalta, Russia; the Island of Rhodes; and Santa Cruz Island of the Galapagos Archipelago. N. multiformis has never been found in the marine environment and cannot be grown in seawater.

The most common nitrite oxidizing bacterium in the oceanic waters and in soils was Nitrobacter winogradskyi (Fig. 8). It was a rod-shaped organism with a polar cap of cytomembranes arranged as flattened vesicles. Strains isolated from the marine and terrestrial environments appeared identical in every respect. Regardless of their source, they

were grown in either freshwater or seawater medium. It seems likely these organisms moved freely between the two environments.

While Nitrobacter winogradskyi was the most common nitrite oxidizer in the waters of the oceans, it was never isolated from marine sediments. Instead we found an entirely new organism in the sediments and have named it Nitrospira immobilis. The cells of this genus were spirally shaped (Fig. 9) and lacked cytomembranes (Fig. 10). Morphologically they resembled the ammonia oxidizer Nitrosospira briensis.

Two additional new nitrite oxidizing bacteria were also discovered in the waters of the ocean during this investigation (Watson and Waterbury, 1971). One of these organisms, Nitrococcus mobilis, was a large coccus (Fig. 11) which had tubular cytomembranes. The other organism was a long thin rod and we called it Nitrospina gracilis (Fig. 12). Nitrococcus mobilis was isolated several times from the Pacific Ocean near the Galapagos Islands and we have also cultured it from beach sand collected in Portugal. Nitrospina gracilis was isolated from the Atlantic Ocean 200 miles east of the mouth of the Amazon River. It did not appear that either of the two latter organisms were as common as Nitrobacter species in ocean waters. Nitrobacter species were the most important nitrite oxidizing organisms in the water column and Nitrospira immobilis was the primary nitrite oxidizer in marine sediments.

IV. Distribution and Number of Nitrifiers

The number of nitrifying bacteria in the terrestrial environment varied from zero to 10^8 cells per gram of soil. In non-fertile

soils the count of nitrifying bacteria was usually small while in agricultural soils, 10^6 - 10^8 nitrifying bacteria per gram were present. The number of nitrifying bacteria in soils stayed relatively constant throughout the year and could account for the amount of nitrification occurring in the terrestrial environment.

During the course of this investigation we made extensive counts of nitrifying bacteria in both inshore and offshore waters using the most probable number technique. For a period of a year weekly samples were taken from Woods Hole Harbor. Large fluctuations in the number of nitrifying bacteria occurred but highest populations were detected in late fall and early winter months. The average number of ammonia oxidizing bacteria counted during the entire sampling period was 413 per liter of seawater and the average number of nitrite oxidizing bacteria was 38.7 per liter. The number of nitrite oxidizers was always significantly less than ammonia oxidizers in both inshore and offshore waters.

We participated in numerous cruises in the Pacific and Atlantic Oceans and in the Black and Red Seas. We examined all depths of the water column and our studies indicated that while nitrifying bacteria were found throughout the water column, they were most prevalent in the upper 200 meters. We never found more than 100 nitrifying bacteria per liter and the average was 2 per liter.

V. Nitrate Utilization and Production in Oceanic Environments

The most accurate estimate of the amount of nitrate used by plants annually can be obtained from temperate and cold northern waters. Here

the surface layers are enriched during the winter by the mixing of the water column to depths of 300-500 meters. Most of our studies indicated that winter mixing results in a surface enrichment of 15 $\mu\text{gm-A}$ of $\text{NO}_3\text{-N/L}$. Clear oceanic waters have a 100 m euphotic zone, thus the phytoplankton will have a reservoir of 1.5 gm-A of $\text{NO}_3\text{-N}$ per m^2 of sea surface. In these rich areas about 200 gms of carbon are fixed by plants/ m^2 /year requiring 2.14 gm-A of nitrogen. The $\text{NO}_3\text{-N}$ available in the upper 100 m of the water column would supply 70 percent of the nitrogen needs of ocean plants.

While it is clear that in temperate and cold northern waters plants use at least 1.5 gm-A of $\text{NO}_3\text{-N}$ per m^2 of sea surface, there is little information concerning the amount of nitrate consumed by plants in the oceans having warm surface waters and a permanent thermocline. The permanent thermocline prevents mixing of the surface waters with the deep nutrient rich waters and consequently, the euphotic zone in these warmer waters is never enriched seasonally with nitrates from the deep waters. It is difficult to estimate how much nitrate is used by plants in these tropical and subtropical oceans.

In all the oceans we estimate that 20 percent of the nitrogen used by plants comes from nitrates and approximately 100 mg-A of $\text{NO}_3\text{-N}$ is used by plants/ m^2 /year. This number is based on Ryther's estimate (1969) that 50 gms of carbon are fixed by plants/ m^2 /year in open ocean waters.

The exact source of nitrates used by plants in the ocean is debatable. Certainly some nitrate comes from terrestrial sources through rain or

river runoff. The amount of nitrates entering the oceans through rain is unknown but in the United States rain supplies 5-8 lbs. of nitrogen/acre²/year. The average rain fall over the oceans is far less than over the continental area of United States, but we shall use the above figure to illustrate the maximum amount of nitrate which could enter the oceanic environment through rain water.

About half of the nitrogen in rain water is in the form of nitrate so we estimate that 2.5 lbs. of NO₃-N fall on each acre of sea surface per year. Using these figures, which are high, the oceans would be enriched with 1.1×10^8 tons of NO₃-N through rain fall annually. This would be equivalent to 16.5 mg -A of NO₃-N/m² of sea surface.

Approximately 3×10^7 tons of nitrogen are fixed commercially per year in the world and an equivalent amount of nitrogen is estimated to flow from terrestrial environments into the oceans. Our studies on the Mississippi River indicated that about 50 percent of the combined nitrogen in the river waters which entered the Gulf of Mexico was in the form of nitrate. If half the combined nitrogen of the entire river runoffs in the world is in the form of nitrate, then 2.21 mg-A of NO₃-N/m² of sea surface/year would be added to the oceans through river runoff. River runoff, even from such nutrient rich rivers as the Mississippi, contribute a small fraction of the nitrogen used by plants in the marine environment. For example, the nitrogen from the Mississippi River would supply only 2 percent of the nitrogen used by plants in the Gulf of Mexico.

From these calculations it is apparent that rain and river waters supply less than 20 percent of the nitrates used by plants in the oceanic environments. At least 80 mg-A $\text{NO}_3\text{-N/m}^2$ of sea surface/year has to be produced in the oceans by the biological or chemical oxidation of ammonia to nitrate. Since no experimental evidence indicates that ammonia is spontaneously oxidized to nitrate in the oceans, we must assume that most of the nitrate in the oceans comes from the biological oxidation of ammonia.

Nitrifying bacteria are primarily responsible for the conversion of ammonia to nitrate in terrestrial environments, and we suspect they serve a similar role in the oceans. However, the supply of nitrate used by plants is far greater than can be produced by the existing number of marine nitrifying bacteria.

Rates of nitrification in the oceans may be estimated from the number of nitrifying bacteria present. Nitrifying bacteria growing under optimal laboratory conditions oxidize 1×10^{-6} $\mu\text{gm-A}$ of $\text{NH}_3\text{-N}$ /cell division. Under these conditions cells divide every 24 hours and a single bacterium could oxidize 3.0×10^{-4} $\mu\text{gm -A}$ of $\text{NH}_3\text{-N}$ /year. The average depth of the oceans is 5,000 m, and if there were two nitrifying bacteria per liter, there would be a total of 3×10^7 nitrifying bacteria under one square meter of sea surface. Under optimal conditions this number of bacteria would oxidize 3 mg-A of $\text{NH}_3\text{-N}$ /year which would supply only 3 percent of the nitrate used by plants. Obviously the nitrifiers in the ocean are not growing under optimal conditions and would produce far less nitrate than estimated above.

We examined the possibility that the primary site of nitrification was in the marine sediments. This may be the case in inshore waters where the water depth is less than 200 m. But in offshore waters we failed to detect the presence of nitrifying bacteria in the sediments. Even in inshore sediments the number of nitrifying bacteria does not exceed $10^7/\text{m}^2$ and this number of bacteria under optimal conditions, could produce only 1 mg-A of $\text{NO}_3\text{-N}/\text{year}/\text{m}^2$ of sea surface.

These studies suggest that rain water, river runoff and nitrifying bacteria contribute less than 25 percent of the nitrates used by plants. It is still unclear how most of the nitrates are produced in the ocean.

In recent studies (Wada and Hattori, 1971) the rates of ammonia oxidation were measured in the upper 100 meters of the water column at two stations in the North Atlantic. In these waters the rate of nitrite production ranged from 10^{-6} to 10^{-4} $\mu\text{gm-A N/liter/hour}$. While ammonia oxidizers appeared responsible for nitrite production, no evidence indicated that nitrite oxidizers oxidized nitrite to nitrate. The maximum rate of ammonia oxidation was found in the nitrite maximum layer at 80 meters. This evidence suggested that the major fraction of the nitrite produced was utilized directly by plants prior to its oxidation to nitrate. It is interesting that we found significantly fewer nitrite oxidizers than ammonia oxidizers in the ocean which may suggest that plants and other microorganisms utilize a significant fraction of the nitrite produced.

Based on the Wada and Hattori report we estimate that at least 2000 nitrifying bacteria per liter were needed to account for the rates of

nitrite production measured in the nitrite maximum layer, assuming that the bacteria were growing under optimal conditions. As previously stated this concentration of nitrifying bacteria is far greater than demonstrated in offshore waters. It seems likely that a group of microorganisms, different from those thus far cultured, are responsible for nitrification in these waters. Additional studies are needed to clarify the inconsistencies surrounding nitrification in the marine environment.

VI. Biochemical Studies

Deoxyribonucleic acid base composition of nitrifying bacteria:

The morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria were compared (Watson and Mandel, 1971, see enclosed reprint). Based on morphological differences, the ammonia oxidizing bacteria were separated into four genera, Nitrosomonas, Nitrosococcus, Nitrosolobus and Nitrosospira. In respect to their G + C compositions, Nitrosomonas species and Nitrosococcus species formed one homogenous group and Nitrosolobus species and Nitrosospira species formed a second homogenous group. Similarly, the nitrite oxidizing bacteria were separated into three genera based on their morphology. The members of the genera, Nitrobacter and Nitrococcus, had similar G + C compositions.

but Nitrospina gracilis had a significantly lower G + C composition than members of the other two genera.

Reductive pentose phosphate cycle in nitrifying bacteria: Assays in cell-free extracts of Nitrosococcus oceanus (Campbell, Hellebust and Watson, 1966) demonstrated the presence of all of the enzymes of the reductive pentose phosphate cycle, with activities high enough to account for the normal growth rate of the cells. Studies on ribulosediphosphate carboxylase activity in the extracts showed that it was inhibited by $MgCl_2$, $MnCl_2$, NaCl and KCl and by sulfate. Phosphate, glutamate, and ethylenediaminetetraacetic acid had no effect. This bacterial enzyme differed from the spinach enzyme with respect to its affinity for bicarbonate and its pH optimum. Whole cells were incubated with $C^{14}O_2$ and the acid-soluble fraction was analyzed by paper chromatography and autoradiography. Phosphoglyceric acid and the sugar phosphates were the earliest labelled compounds; several amino acids and organic acids were also labelled. It was concluded that N. oceanus incorporated CO_2 primarily via the reductive pentose phosphate cycle and that the mechanisms of CO_2 fixation were similar to that found in most plants.

Ammonia oxidation in cell-free extracts: The oxidation of ammonia to nitrite involves the removal of six electrons but little is known about the metabolic pathway of this oxidative reaction. For many years it had been proposed that hydroxylamine was one of the intermediate products as both cell-free extracts and whole cells could oxidize hydroxylamine to nitrite. However, previous investigators failed to demonstrate the

oxidation of ammonia to nitrite in cell-free extracts. We demonstrated ammonia oxidase activity in cell-free extracts of Nitrosococcus oceanus (Watson, Asbell and Valois, 1970, see enclosed reprint). Ammonia and hydroxylamine were oxidized at approximately the same rates in these cell-free systems and this observation tended to verify that hydroxylamine may be an intermediate between ammonia and nitrite. This enzyme system was particulate and probably membrane bound.

The enzyme or enzymes involved in the oxidation of ammonia to hydroxylamine were extremely labile and inhibited by minute quantities of peroxides. As little as $10^{-8}M$ hydrogen peroxide provided a 50 percent inhibition of this reaction. Continuation of this study should resolve the intermediates involved in the oxidation of ammonia to nitrite.

Fatty acid composition of nitrifying bacteria: Nitrifying bacteria differ from most other bacteria by possessing a complex cytomembrane system. We were interested in determining if this morphological complexity could be correlated with an unusual fatty acid composition. Fatty acids in the lipids of 19 marine and terrestrial nitrifying bacteria were analyzed (Blumer, Chase and Watson; 1969). The analysis showed that ammonia oxidizing bacteria have the simplest fatty acid composition of any group of bacteria other than the mycoplasmas. Palmitic and palmitoleic acids accounted for 96-100 percent of the total fatty acids. The fatty acids of the nitrite oxidizing bacteria covered a wider range from C_{14} to C_{19} , but from two to four fatty acids still accounted for more than 80 percent of the total acids. Traces of branched iso- and anteiso-acids

were present in only two of the 19 bacteria.

Basis of autotrophy in nitrifying bacteria: Enzymatic assays of cell-free extracts of the ammonia oxidizing bacterium Nitrosococcus oceanus established that obligate autotrophy was not the result of the lack of any given enzyme system (Williams and Watson, 1968, see enclosed reprint). All of the Embden-Meyerhoff enzymes except phosphofructokinase, and all of the tricarboxylic acid cycle enzymes, as well as reduced nicotinamide adenine dinucleotide oxidase, were found in these extracts. While all of the tricarboxylic acid cycle enzymes were present, the specific activities of α -ketoglutarate dehydrogenase and succinic dehydrogenase were so low that it seemed unlikely the organisms could cycle organic molecules rapidly enough to supply the energy needs of the cell. A phosphoenolpyruvate- CO_2 -fixing system was also demonstrated. Resting cells incubated with C^{14} -D-glucose and C^{14} -L-glutamate and cells grown in the presence of C^{14} -labelled glucose, glutamate, pyruvate and methionine incorporated these compounds into cellular material, but at a level too low to provide the cell's major carbon and energy needs. For example, for every molecule of pyruvate that the cells obtained from external sources, they formed 1000 molecules of pyruvate by the fixation of CO_2 .

VII. Morphology of Nitrifying Bacteria

Cell walls of Nitrosococcus oceanus: We examined the fine structure of the cell walls of all the nitrifying bacteria but studied the wall of

Nitrosococcus oceanus (Watson and Remsen, 1970, see enclosed reprint) in detail. N. oceanus, like other nitrifying bacteria, is Gram-negative. In thin section the cell envelopes of most Gram-negative bacteria consist of an outer and inner double track layer, each about 80 Å wide, separated by an area of low electron density about 100-200 Å wide. This intermediate layer, often referred to as the periplasmic space, is usually bisected by a 20 Å dense layer which is considered the site of peptidoglycan synthesis. In some cases the outermost double track layer, composed of lipopolysaccharides, is associated with macromolecular subunits ordered in a regular array.

The cell envelope of N. oceanus was composed of eight distinct layers designated as the W-1 to W-8 layers (Fig. 19). The outermost layer, W-1, was composed of slime fibrils (Fig. 13). This layer was not a permanent feature of the cell envelope and was frequently absent. The W-2 layers lay just beneath the W-1 layer and was composed of 50 Å subunits arranged in an hexagonal array (Fig. 14 and 15). These subunits had a periodicity of 120 Å. An overall view of a frozen etched cell revealed that this layer, while covering the entire cell, was lapped and not continuous over the entire surface (Fig. 14). Beneath the 50 Å layer lay another layer composed of subunits arranged in a molecular array. This layer, (W-3), composed of 40 Å subunits, was easily seen in both freeze-etch (Fig. 14) and negative-stain preparations (Fig. 16). In cross-sections (Fig. 19) it was not apparent that the W-2 and W-3 layers were distinct from each other.

The W-1, W-2 and W-3 layers were usually missing in the cell envelopes of other Gram-negative bacteria. While the cell envelope of N. oceanus appeared unique in possessing these additional layers, the remainder of the cell envelope was multilayered and resembled most other Gram-negative bacteria. The lipopolysaccharide layer (W-4), which acts as the outer boundary in most cell envelopes, was located just beneath the W-3 layer in N. oceanus. This double track layer was seen in thin section (Fig. 19) but was not seen in freeze-etch preparations as it was not fractured during the freeze-etch process.

In thin sections (Fig. 19) the area between the lipopolysaccharide layer (W-4) and the plasma membrane (W-8) was normally electron transparent. Occasionally it was bisected by a 20 Å dense line believed to represent the peptidoglycan component (W-6) of the cell envelope. Thin sections normally revealed little structural detail of the portion of the cell envelope lying between the W-4 and W-8 layers. However when the cell envelope was freeze-fractured, a fracture plane occurred at the level of the peptidoglycan revealing that the electron transparent areas on each side of the peptidoglycan layer were composed of globular subunits. As the fracture plane occurred at the level of the peptidoglycan layer, the concave surface of the upper globular layer (W-5) (Fig. 17) and the convex surface of the lower globular layer (W-7) (Fig. 18) were exposed. The W-5 globular layer was bound closely to the W-4 lipopolysaccharide layer while the W-7 layer appeared closely associated with the plasma membrane (W-8). The chemical nature of these two globular layers is not known, but probably they are composed of lipoproteins. The plasma membrane (W-8) seen in thin

sections (Fig. 19) appeared as a double track layer and was similar in appearance to the plasma membrane of other bacteria.

Cell walls of marine Nitrosomonas species: As previously indicated, the only ammonia oxidizers found in the oceans belonged either to the genus Nitrosococcus or Nitrosomonas. Although the cell walls of the Nitrosomonas were not as complex as those of Nitrosococcus oceanus, they were morphologically different from those of the terrestrial species in this genus. The cell envelopes of both terrestrial and marine species of Nitrosomonas were composed of multiple layers and generally resembled other Gram-negative bacteria. The chief difference between the cell envelopes of marine and terrestrial species was that the marine bacteria possessed an additional cell wall layer (Watson and Remsen, 1969, see enclosed reprint). Two different molecular patterns were found in the marine strains. In most strains the additional cell wall layer was composed of 50 Å subunits arranged in a hexagonal pattern (Fig. 4) similar to that found in Nitrosococcus oceanus. Several strains of marine ammonia oxidizers lacked this pattern but had instead an additional cell wall composed of large subunits, approximately 150 Å in diameter (Fig. 3).

The sculptured, outer wall layer of these marine ammonia oxidizing bacteria is believed to be composed of structural proteins, with the protein units linked together through metal-oxygen bonds. This theory is based on evidence that the outer layer was unaffected by hydrogen-bond breakers, such as urea, urethane, acetamide, formamide, and glycolic acid, but was broken down when treated with formic acid, a known metal-oxygen bond breaker. In addition, this outer layer of repeating subunits

was almost completely removed from the cell wall when treated with ethylenediaminetetraacetate, which chelates metal ions. The metals involved and the method of linkage are at present unknown; however, it seems likely Ca^{++} or Mg^{++} would be present since they are known constituents of cell walls.

The effect of this additional cell wall layer on the ecological role of marine ammonia oxidizers was not established. It probably provides some structural rigidity to the cells and may prevent attachment of viruses and parasitic bacteria by masking the lipopolysaccharide layer. Whether many other marine bacteria possess similar additional cell wall layers was not determined. We found morphologically similar cell wall fragments (Degens, Watson and Remsen, 1970) in sediments from the Black Sea which were 3000-7000 years old. The high content of heavy metals in these sediments provided a sterile environment, preserving these bacterial fragments from decomposition for several thousand years.

Cell walls of terrestrial ammonia oxidizers: Most of the terrestrial ammonia oxidizers had a typical Gram-negative cell envelope and will not be detailed in this review. The most unusual cell wall in the terrestrial genera was found in Nitrosolobus multiformis. As previously indicated the cytoplasm of this lobular organism was partially compartmentalized by the intrusion of cytomembranes (Fig. 7). Several genera of the ammonia oxidizers had cytomembranes which appeared to arise through the invagination of the plasma membrane. In most bacteria the intrusion of the plasma membrane into the cytoplasmic regions of the cells was not accompanied by

the invagination of other components of the cell envelope. In Nitrosolobus multiformis the invaginating plasma membrane was accompanied by a similar invagination of the peptidoglycan layer and one or both of the globular layers of the cell envelope. By the invagination of additional cell envelope components, the cells have structurally stabilized their cytoplasmic compartments. This structural adaptation is unique among bacteria and, as far as we know, is only present in Nitrosolobus multiformis.

Cell walls of nitrite oxidizing bacteria: All four nitrite oxidizing bacteria studied were Gram-negative and two of these organisms, Nitrospina gracilis and Nitrococcus mobilis, had typical Gram-negative cell envelopes. The two other nitrite oxidizers, Nitrobacter winogradskyi and Nitrospira immobilis had unique features in their cell envelopes which set them apart from other Gram-negative bacteria. Nitrobacter winogradskyi did not possess the usual outer double track lipopolysaccharide layer. Instead the cell was circumvallated by a cell wall layer, the inner portion of which stained heavily and the outer portion of which remained almost electron transparent (Fig. 20). The chemical composition of this outer cell wall component is still unknown and we are now trying to determine if it is comprised of a lipopolysaccharide.

Nitrospira immobilis had an unusual cell envelope in that the periplasmic space was 300-400 Å wide (Fig. 21). This width was twice that found in most Gram-negative bacteria. Usually the material occupying this space in Gram-negatives does not stain, making this space electron

transparent. In Nitrospira immobilis the periplasmic material took up stain readily and was always electron dense. In some cells the material in the periplasmic space formed cancerous-like protrusions (Fig. 22).

The nitrifying bacteria comprise a relatively small group of organisms. The bacteria in each physiological group were almost physiologically indistinguishable from one another. Yet morphologically, the cell envelopes demonstrated a wide spectrum of morphological diversity, a most unusual trait for any group of bacteria. This diversity offers a unique system to those interested in structure-function relationships.

Membranes of nitrifying bacteria: Membranes fulfill a variety of functions and are needed by all living cells. In most procaryotic organisms the cells only have a plasma membrane but in eucaryotic cells specialized membranous organelles are also present. It is generally assumed that membranous organelles in eucaryotic cells were initially formed by the invagination of the plasma membrane. If these membranous organelles in higher cells originated in this way, one might expect to find some procaryotic cells in which the cytoplasmic membrane has invaginated to form a primitive type organelle. Nitrifying bacteria are one of the few groups of bacteria in which transitional changes in membrane organization can be seen.

We can only speculate on the physiological need of nitrifying bacteria which initially triggered the invagination of their plasma membrane. Possibly more membranes were needed to house additional enzymes or perhaps the plasma membrane was invaginated forming cytoplasmic canals to expedite

the entrance and removal of substrates and end products in and out of the cell. If the latter was the case, then the extent and arrangement of these infoldings should be influenced by the size and shape of the cell. On this basis one would predict that the most extensive invaginations would be found in those cells having the lowest surface area/volume ratio. It would also be anticipated that one would not find extensive invaginations of the plasma membrane in cells with a large surface area/volume ratio. Both these conditions could be demonstrated in nitrifying bacteria. In the long slender cells of Nitrospina gracilis (Fig. 12), Nitrosospira briensis (Fig. 6) and Nitrospira immobilis (Fig. 10) no invaginations were evident. In the shorter fatter cells of Nitrosomonas and Nitrobacter species which have a decreased surface area/volume ratio, considerable invaginations of the plasma membrane forming peripheral cytoplasmic canals were seen. In the large cells the surface area/volume ratio was lowered even further and more extensive invaginations were evident in the cells of Nitrosococcus oceanus (Fig. 1), Nitrococcus mobilis (Fig. 11) and Nitrosolobus multiformis (Fig. 7). The cytoplasmic canals in these larger cells penetrated deep into the central regions of the cell.

Cytoplasmic canals would function more efficiently if they could contract and expand in a pumping-like action. Contraction and expansion of the cytoplasmic canals was found in Nitrosococcus oceanus (Fig. 23, 24) and these conformational changes could be experimentally controlled. Energy and metal ions were required to maintain these canals in the

contracted state. When the cells were deprived of an energy source or when ions were removed by EDTA, the lumen of the cytoplasmic canals expanded. Whether other nitrifying bacteria exhibit similar conformational changes was not experimentally verified.

As indicated, the primary reason for the infolding of the plasma membranes was to increase membrane surface area or to accelerate the movement of substrates and end products in and out of the cell. Since these infolded membranes can be disposed in a variety of ways, one could anticipate that one or more arrangements would compartmentalize the cell. This compartmentalization was observed in Nitrosolobus multiformis (Fig. 7). Certainly compartmentalization in N. multiformis is not as advanced as that found in eucaryotic cells, but even in this bacterial cell physiological compartmentalization accompanied the morphological differentiation of the cytoplasm. For example, glycogen was deposited only in the peripheral and not in the central compartments (Fig. 25). A similar primitive, and possibly accidental, compartmentalization of cells may have occurred during the transition of procaryotic to eucaryotic cells.

In Nitrosolobus multiformis strands of DNA were seen in all compartments suggesting that pores existed between compartments. These pores were rarely visible in sectioned material but we feel many were masked by the thickness of the section.

If primitive eucaryotic cells were compartmentalized, DNA and ribosomes would be trapped in these specialized areas. Possibly DNA and ribosomes in these compartments evolved

independently of similar components in the rest of the cell. It is possible that each isolated compartment underwent further biochemical and morphological specialization to form membranous organelles such as chloroplasts or mitochondria. The above sequence would provide a mechanism for the evolution of membranous organelles and could account for the development of satellite DNA and the different ribosome class associated with these organelles. Another common belief is that chloroplasts and mitochondria arose from a symbiotic relationship between procaryotic and eucaryotic cells. This method of development should also be considered.

Normally the cell wall components of most Gram-negative organisms are restricted to the outer most regions of the cell and do not invaginate into the cytoplasm. One of the cell wall components is the peptidoglycan layer which provides rigidity to the cell. If this layer accompanied the invagination of the plasma membrane it could provide rigidity to the cellular compartments. In Nitrosolobus multiformis the peptidoglycan layer and one of the globular layers of the cell wall invaginated with the plasma membrane into the cytoplasm creating stable compartments within the cell. To our knowledge this is the first time this phenomenon has been reported.

Conformational changes in membranes of bacteria must be accompanied by compositional changes. A comparison of the biochemical and fine structure of the membranes in various species should help delineate some of the molecular transitions which took place as membrane systems

evolved. Preliminary compositional studies were initiated using Nitrosococcus oceanus, Nitrobacter winogradskyi and Nitrococcus mobilis.

A fundamental question asked concerning the composition of the membranes was whether the outer and inner surfaces were identical. In Nitrosococcus oceanus the fact that the outer surfaces of membrane vesicles fuse into a "zipper-like" junction forming a tripartite structure (Fig. 23), while their inner surfaces do not, suggested that the membranes were asymmetrical. The two membranes, comprising the tripartite structure of the "zippered-junction", separated when treated with trypsin, lipase or mechanical forces. This indicated that the outer surfaces were faced with lipids and proteins involved in the linkage of adjacent membranes.

High concentrations of ammonia and nitrite are toxic to most bacteria. Nitrifying bacteria live in high concentrations of these substrates and the cells must provide some mechanism to prevent the entrance of toxic substances into the cytoplasm. If the enzymes responsible for the oxidation of the substrates were on the outer portion of the plasma membrane or the inner surface of the membranes of the cytoplasmic canals, the substrates could be oxidized without entering the cytoplasm. We found some evidence to verify this hypothesis. We know the enzymes were membrane bound and that membrane vesicles were formed at the time of cell rupture. Membranes prepared from cells ruptured with the French press oxidized ammonia to nitrite while those prepared by means of the Braun cell disintegrator remained in their

natural state. This suggests that the enzymes on the vesicles in the French press preparations were on the outer surface of the vesicle and exposed to the substrate while the enzymes in the Braun preparation were on the inner surface of the vesicle membrane and not in contact with the substrate. This evidence argues for the asymmetry of membranes.

Even more convincing evidence that membranes are morphologically asymmetrical was found in Nitrobacter winogradskyi. When these cells were sectioned and examined at high magnification (Fig. 26), it was apparent that the cells had three sets of flattened vesicles and that each vesicle had an open electron transparent lumen. The outer surface of these vesicles were covered with projecting particles which formed an electron dense layer 90-100 Å wide. The inner surface of these vesicles was void of particles. In negatively stained membranes the particles were found to exist in square packets about 130 Å wide and each packet was composed of four subunits 65 Å in diameter (Fig. 27). These particles were arranged in one or two directions. In freeze-etch preparations these subunits had a diameter of 60 Å and were queued in rows 120-160 Å wide (Fig. 28). The structural and functional significance of these particles was not investigated.

The surface structure of the membranes in Nitrosococcus oceanus was also examined. When the membranes from this organism were negatively stained, the outer surface of the vesicle was found covered with hexagonally arrayed 80 Å "doughnut" shaped particles (Fig. 29), separated by a space of 10-15 Å. Each particle was composed of four 40 Å subunits...

These particles and subunits are believed to be structural or functional components of the membrane rather than protrusions from it as was found in Nitrobacter winogradskyi. This was shown by the fusing of the adjacent membranes which formed a tripartite structure that was never more than twice the width of a single membrane. When cells were ruptured with a French press, the hexagonal arrangement disappeared indicating that the particles were held together by weak bonding forces. These particles were separated from an underlying smooth layer by sonication suggesting again a weak bond between these layers. On the other hand, the 40 Å subunits of the membrane were not disassociated by hydrogen bond breakers such as urea or by mechanical means indicating a relatively strong bonding between subunits. Possibly when conformational changes occurred the "doughnut" shaped particles were rearranged and the individual "doughnut" shaped particles of four 40 Å subunits were not morphologically altered.

The fine structure of membranes was studied using the freeze-etching technique. This technique showed basic differences between the plasma membrane and cytomembranes. The fracture face exposed in the plasma membrane was composed of smooth islands about 200 Å in diameter surrounded by loosely packed 80 Å particles. In contrast, the fracture face of cytomembranes exposed revealed a series of high and low smooth plaques about 200 Å in diameter and relatively few particles (Fig. 30). This image was produced by the fracture plane alternating between two surfaces approximately 60 Å apart. It seems likely that the two adjacent plaques were revealing the hydrophobic region and the surface

of membranes. The prominent white areas seen in Fig. 30 represent the lumen of the vesicles. Adjacent to these white areas were relatively smooth small patches which showed the surface of the membranes exposed by deep etching.

During the freeze-etching procedure, membranes should fracture along a plane where the bonding forces are the weakest. The weakest bonding forces exist between the hydrophobic tails of the hydrocarbons. If the central core of the membranes was composed of a continuous bilayer of lipids, the fracture would split the membrane and only one fracture plane would be apparent. The fact that two fracture planes were evident in both the plasma membrane and cytomembranes tends to refute the idea that a continuous bilayer of lipid existed in these membranes. This evidence prompts us to theorize that the lower fracture plane seen in the cytomembranes occurred between the hydrophobic tails of the hydrocarbons and that the upper fracture plane occurred along the membrane surface. Thus the large smooth patches seen in freeze-etch preparations of membranes represented the central hydrophobic region of the membrane while the upper fracture plane which produced evidence of particles occurred along the surface of the membrane.

An alternation of the fracture plane suggested that intramembranous bridges extended into and interrupted the bimolecular leaflet of lipids. We believe that these bridges were composed of subunits which contained enzyme complexes responsible for the transport of material across the membrane surface. These intramembranous bridges were more common in the

cytomembranes than in plasma membranes and occupied about 50 percent of the area in the core of the membranes.

We briefly examined the tubular type membranes found in the nitrite oxidizer, Nitrococcus mobilis (Fig. 11). When cells of N. mobilis were ruptured and negatively stained (Fig. 31), branched membranous tubes covered with 80 Å particles were evident. These particles usually appeared randomly arranged but evidence that they were in an ordered array was observed in a fortuitous tangential section (Fig. 32). As in Nitrosococcus oceanus the 80 Å particles appeared to be part of the membrane rather than projections from the membrane as seen in Nitrobacter winogradskyi. The "doughnut" shapes appeared to be weakly bound to each other and capable of rearranging as observed in Nitrosococcus oceanus. In one conformational state these particles were arranged in a hexagonal pattern but it was obvious that particles were being rearranged as conformational changes took place.

The nitrifying bacteria are a unique group of organisms which provide an opportunity to follow morphological and biochemical events which occurred in membrane evolution. Since the organisms in each physiological group perform biochemically identical functions, it should be possible to differentiate between structural and functional subunits and to define the morphological and biochemical differences existing between membranes.

Nitrifying bacteria may serve as more convenient tools for the study of membrane structure than cells from higher organisms. Membrane

isolation can be affected more easily and with less stress from nitrifying bacteria than from eucaryotic cells. In contrast to many eucaryotic cells, the external environment of the nitrifiers can be varied and the factors which influence membrane synthesis and structure can be studied.

Since the terrestrial and marine nitrifying bacteria appeared physiologically identical, with the exception of their ion requirement, we hope to use these organisms to learn more about the differences between membranes of marine and terrestrial organisms.

VIII. Microbial Decomposition of Organic Compounds

Urea: During the last two years we examined the possibility that urea might serve as an important plant nutrient in the oceans. We studied urea decomposition off the west coast of Africa and in the waters around Woods Hole, Massachusetts. On the continental shelf the concentration of urea varied from 2-3 $\mu\text{gm-A urea-N/liter}$. While no evidence was found that the urea concentration varied significantly with depth in inshore waters, a marked difference was found in urea concentrations in off-shore waters between the upper 100 meters and the rest of the water column. In the upper 100 meters there were 2-3 $\mu\text{gm-A urea-N per liter}$ but below this depth the concentration dropped sharply to below 1 $\mu\text{gm-A urea-N/liter}$. Since no sharp concentration gradients were found within the euphotic zone, urea did not appear to be utilized directly by off-shore plants. As little urea was found below 100 meters, the animals responsible for urea production must live primarily in the upper 100

meters of the water column.

Other studies indicated that most of the offshore phytoplankton did not utilize urea directly as a nitrogen source. In offshore waters urea must first be broken down to ammonia before it can be utilized by plants. On the African cruise we estimated the rates of urea breakdown in the ocean by adding 2 $\mu\text{gm-A}$ of C^{14} labelled urea-N/liter to seawater. Samples were collected every 5-10 meters down to a depth of 200 meters and then incubated four hours at in situ temperature. The evolved C^{14}O_2 from the urea breakdown was trapped and counted. The rate of urea decomposition was determined in 100 water samples from the continental shelf of Northwest Africa where the average depth was 200 meters. The rate of urea breakdown in these samples varied from 1×10^{-5} to 2×10^{-3} n moles of urea/hour/ml and averaged 4×10^{-4} n moles of urea/hour/ml. Based on this rate, we estimate that 1.4 gm-A of urea-N/m² would be broken down annually and released in the form of $\text{NH}_3\text{-N}$ which would be available for plant growth.

In these waters approximately 100 grams of carbon are fixed per m² of sea surface per year and this would require 1.07 gm-A of N per m² of sea surface per year for this amount of plant growth. These studies suggest that most of the nitrogen used by plants in these inshore waters would be cycled through urea and that the rate of urea breakdown would dictate the rate of plant production in these waters. Whether urea plays a similarly important role in other oceanic areas was not determined.

The rate of urea decomposition in Woods Hole waters varied from

1.3×10^{-5} to 2.3×10^{-3} n moles/hr/ml. The lowest values were obtained in November and the rates gradually increased with the highest values found in the month of January. The rate decreased slightly during the middle of February but increased during March and April to values comparable to those found in January. In inshore waters many of the phytoplankton species were capable of breaking down urea. The rate fluctuations may have reflected changes in the phytoplankton population during this period.

The number of urea decomposing bacteria in both inshore and offshore waters varied greatly. The average number of urea decomposing bacteria in offshore waters was 3×10^4 /liter and in inshore waters 1.64×10^5 /liter. Similarly the rates of urea decomposition were approximately one order of magnitude higher in inshore than in offshore waters.

Glucose and acetate oxidation and incorporation in inshore waters:

Bacterial glucose and acetate incorporation and oxidation in local Woods Hole waters was measured. No attempt was made to determine in situ rates. For these experiments local water samples were collected weekly and enriched either with 1 μ mole of C^{14} labelled acetate or glucose. The samples were incubated for four hours and the amount of C^{14} uptake in cells and the $C^{14}O_2$ released was determined. The rates of glucose and acetate oxidation was 3.8×10^{-3} n moles/hr/ml. The rates of acetate and glucose incorporation varied from 5×10^{-4} to 1.2×10^{-2} n moles/hr/ml. The average rate of acetate incorporation was 4×10^{-3} n moles/hr/ml. About 50 percent of the organic compounds

used by bacteria was oxidized to CO_2 and about 50 percent incorporated into the cells.

Based on these rates the bacteria in these waters would oxidize and incorporate about 1.0 gm-A of acetate-C and about 1.5 gm-A of glucose-C/ m^2 of sea surface/year. The total carbon oxidized represents only a small fraction of the total carbon fixed by plants per year in these waters.

IX. Endotoxin Studies

During the last year we embarked on a new study involving bacterial lipopolysaccharides, also known as pyrogens or endotoxins. Nearly all Gram-negative bacteria are cloaked with an external layer of lipopolysaccharides which appears as a double track layer in thin section. The medical significance of these bacterial lipopolysaccharides was recognized even prior to the turn of this century when it was discovered that sterile saline or sterile distilled water contained a heat stable compound capable of inducing fever and other toxic reactions in humans. These heat stable compounds were subsequently discovered to be small fragments of Gram-negative bacterial cell walls composed of lipopolysaccharides and were called endotoxins or pyrogens.

These bacterial lipopolysaccharides are ubiquitous and are not destroyed at temperatures normally used for sterilization. This presents a problem in the manufacture of drugs which are used for intravenous injection.

Endotoxins play another equally important role in the medical field. Although relatively abundant in the human intestine, endotoxins remain innocuous as long as they are prevented from entering the blood stream by the permeability barrier of the intestinal mucosa. If this permeability barrier is destroyed by trauma or disease, the endotoxins enter the blood stream and death can result. Endotoxins also gain entrance into the blood stream through Gram-negative bacterial infections, and in these cases endotoxemia may be the major cause of death.

In spite of the medical importance of endotoxins no good quantitative test has been developed for their detection. A number of investigators during the past few summers at Woods Hole have developed a test for the detection of endotoxins (Levin and Bang, 1964; Rojas-Corona et al., 1969; Reinhold and Fine, 1971; Levin et al., 1970). For this test amebocytes from the horseshoe crab (Limulus polyphemus) are collected from the hemolymph, pelleted by centrifugation and lysed either in distilled water or in Tris buffer. This lysed fraction is centrifuged and its supernatant is saved and called the Limulus lysate. This material may be stored in a frozen state for a year or more without loss of activity.

The presence of endotoxins is measured by mixing equal quantities of endotoxin and lysate and incubating the mixture at 37°C for an hour or more. The presence of endotoxin causes this mixture to clot. Normally the endotoxins are serially diluted and 0.1 ml of each dilution is mixed with an equal quantity of lysate. The endpoint of the test is read as the highest

dilution of endotoxin resulting in the formation of a clot. Most endotoxins tested will form a clot when as little as 10^{-3} $\mu\text{gm/ml}$ is mixed with the Limulus lysate. In highly purified endotoxins we have obtained titers of 10^{-6} $\mu\text{gm/ml}$.

We believe this test can be used in seawater to determine the concentration of free bacterial lipopolysaccharides and the total bacterial biomass in any sample of seawater. To perform this test a seawater sample will be filtered through a Millipore filter. The filtrate will be tested for free bacterial lipopolysaccharides and the bacterial cells on the Millipore filter will be treated with a phenol-water mixture to extract the lipopolysaccharides. The lipopolysaccharides will be contained in the water phase of this mixture which will be dialyzed against distilled water to free the sample of phenol. After dialysis the concentration of lipopolysaccharides in this sample will be quantitated by means of the Limulus lysate test.

We are now in the process of developing this test. In seawater collected from the Woods Hole area we can obtain a positive test when the seawater is diluted 1000 times. This would indicate that the concentration of free bacterial lipopolysaccharides is 1 $\mu\text{g/ml}$ in these samples. Offshore samples thus far have not been tested.

In cultures of Escherichia coli we can detect as few as 1000 bacteria with this method. We are still testing cultures to perfect our technique but shall try to estimate the seawater bacterial

biomass in the near future with the Limulus test. We believe that this can be used for bacterial biomass determination since bacterial lipopolysaccharides found only in Gram-negative bacteria constitutes about ten percent of the dry weight of the cell. Over ninety-nine percent of the bacterial biomass in the ocean is composed of gram-negative bacteria. Since the test is specific for bacterial lipopolysaccharides, the bacterial biomass can easily be distinguished from the biomass of other organisms.

At present no method is available to determine the total bacterial biomass in seawater. Using standard plate count procedures only one percent of the total bacterial population can be counted at a given time. Direct counts of bacteria are equally unsatisfactory. If the Limulus lysate test can be perfected as we believe it can, it will be a valuable method for studying the natural distribution of bacteria and bacterial lipopolysaccharides in seawater. Multiple applications of this test are immediately apparent. For example, the test could be used to determine the degree of pollution or to determine the effects of a pollutant on a bacterial biomass. It should also be possible to quantitate the number of bacteria in sediments with this technique. The lipopolysaccharides in sediment samples will be extracted with the phenol-water mixture and the lipopolysaccharide concentrations determined with the Limulus lysate.

This summer we are collecting blood and preparing the Limulus lysate for our future studies. Since each assay requires a 1:1 ratio of lysate to sample, we hope to collect at least four liters this summer which will permit us to initiate our testing program in the fall and

winter months. This summer we also have initiated biochemical studies on the active factor in the lysate in an attempt to purify and identify this compound.

This test may be of additional interest to the Atomic Energy Commission. It is our understanding that some radioisotopes with extremely short half lives are used for the treatment of certain diseases in humans. If such radioisotopes are contaminated with endotoxins and if these isotopes are injected into patients there is always the risk that the endotoxins could be fatal to the patient. The rabbit fever test cannot be used to detect the presence of endotoxins because of the short half life of the radioisotope. With the Limulus test one can determine within 30 minutes whether the radioisotope is contaminated with endotoxin.

When perfected, this test will be able to detect endotoxemia in patients, determine bacterial biomass in water samples, define the action of endotoxins in humans and may also be used to study basic clotting mechanisms in humans. Because of the great potential of this test, we feel that the time spent on this project is fully justified.

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XI. Publications in Last Five Years

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XII. Figure Legends and Figures

- Figure 1. Thin section of Nitrosococcus oceanus showing cytomembranes arranged as flattened lamellae in the central region of the cell. X 108,000.
- Figure 2. Thin section of a marine strain of Nitrosomonas, shows cytomembrane arranged as flattened lamellae in peripheral region of the cell. X 68,000.
- Figure 3. Freeze-etching of a marine strain of Nitrosomonas showing an outer cell wall layer composed of repeating subunits. 92,000.
- Figure 4. Freeze-etching of another marine strain showing outer cell wall layer composed of subunits arranged in a hexagonal array. X 89,500.
- Figure 5. Negative stain of Nitrospira briensis showing spiral nature of cells. X 48,000.
- Figure 6. Section of Nitrospira briensis showing general morphology and lack of cytomembranes. X 63,000.
- Figure 7. Section of Nitrosolobus multiformis showing lobular shape of cells which are partially compartmentalized by the intrusion of membranes into the cytoplasm. X 48,000.
- Figure 8. Section of Nitrobacter winogradskyi showing polar cup of cytomembranes. X 118,000
- Figure 9. Negative stain of Nitrospira immobilis showing spiral nature of cells. X 45,000.

- Figure 10. Cross section through one part of a spiral of Nitrospira immobilis showing lack of cytomembranes. 126,900.
- Figure 11. Section of Nitrococcus mobilis showing general morphology and tubular cytomembranes. X 43,000.
- Figure 12. Section of Nitrospina gracilis showing shape of cells and lack of cytomembranes. X 57,500.
- Figure 13. Freeze-etching of Nitrosococcus oceanus showing outermost (W-1) cell wall layer. X 830,000.
- Figure 14. Freeze-etching of Nitrosococcus oceanus showing cell wall layers W-2 and W-3. W-2 layer composed of 50 Å subunits arranged in a hexagonal pattern and the W 3 layer is composed of 40 Å subunits arranged in a rectilinear array. X 130,500.
- Figure 15. Negative stain of the W-2 cell wall layer N. oceanus showing arrangement of 50 Å subunits. X 640,000.
- Figure 16. Negative stain of the W-3 cell wall layer of N. oceanus showing arrangement of 40 Å subunits. X 107,000.
- Figure 17. Freeze-etching preparation of N. oceanus showing a concave view of the globular W-5 cell wall lipoprotein layer. X 271,000.
- Figure 18. Freeze-etching preparation of N. oceanus showing a convex view of the globular W-7 cell wall lipoprotein layer. X 144,000.
- Figure 19. Section of cell envelope of N. oceanus showing W-2, W-3, W-4, W 5, W-6, W-7 and W-8 layers. In this preparation it is not possible to distinguish between the W-2 and W-3 layer. The

W-4 lipopolysaccharide layer has a double track appearance, the W-5 and W-7 layers are not stained in this preparation, the W-6 layer is composed of peptidoglycan and the W-8 layer is the plasma membrane.

- Figure 20. Section of the cell wall of Nitrobacter winogradskyi. Note the lack of a double track outer cell wall layer. Instead the outer most layer is almost electron transparent and just beneath this layer is another layer which stains intensely X 415,000.
- Figure 21. Section of the cell envelope of Nitrospira immobilis. Note that the width of the periplasmic space (P) is more than double that of most Gram-negative bacteria. X 559,000.
- Figure 22. Section of the cell envelope of Nitrospira immobilis showing how the material in the periplasmic space has formed a cancerous type growth. X 150,000.
- Figure 23. Section of the cytoplasmic membranes in Nitrosococcus oceanus in a conformational state where the lumen of the vesicles are nearly closed. X 288,000.
- Figure 24. Section of the cytoplasmic membranes in N. oceanus revealing conformational state where lumen of vesicles are expanded. X 942,000.
- Figure 25. Section of Nitrosolobus multiformis stained with a stain specific for glycogen. Predominance of glycogen (G) in the peripheral compartments suggest this bacterium has

physiological as well as morphological compartmentalization.

X 75,000.

- Figure 26. Section of Nitrobacter winogradskyi showing the asymmetry of membranes. Note the darkly stained material projecting from the outer surface of the cytoplasmic vesicles. X 543,000.
- Figure 27. Negative stain of a cytomembrane of Nitrobacter winogradskyi revealing the particles which line the outer surface of the vesicles. X 272,000.
- Figure 28. Freeze etching of an isolated membrane of Nitrobacter winogradskyi. Fracture plane has passed along surface of membrane thus revealing arrangement of particles shown in Figure 26 and 27. X 334,000.
- Figure 29. Negative stain of Nitrosococcus oceanus membranes revealing hexagonal arrangement of $80 \text{ \AA}$ particles. X 335,000.
- Figure 30. Freeze etching of Nitrosococcus oceanus showing how fracture plane has alternated between the surface and the internal region of the membrane. X 166,000.
- Figure 31. Negative stain of Nitrococcus mobilis tubular membranes showing that these membranes are composed of $80 \text{ \AA}$ particles. X 335,000.
- Figure 32. Tangential section of membranes of Nitrococcus mobilis membranes showing how particles (P) are arranged in a regular array in these membranes. X 240,700.

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