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I. Abstract

These studies have dealt with the role bacteria play in the mineralization of plant nutrients in the ocean. A new method based on the Limulus lysate test for the detection of Gram-negative lipopolysaccharides was developed and utilized to determine the horizontal and vertical distribution of bacteria in the oceans. This test was used in conjunction with the ATP method to determine what percentage of the total biomass in oceanic environments was comprised of bacteria. The biochemical events occurring when the Limulus lysate was reacted with Gram-negative lipopolysaccharides were investigated. The role of the nitrogen-fixing bacteria in oceanic waters was examined.

II. Technical Report

These studies have been concerned with the role bacteria play in the nitrogen cycle, the mineralization of organic matter and the recycling of plant nutrients in the oceans. They were aimed at determining how much of the organic matter in the ocean is oxidized by the bacteria and how much is utilized by higher organisms. One of the major objectives was to develop a new technique to quantitate the bacterial biomass in oceanic environments.

Bacterial Biomass Determinations

Background Information: In the ocean bacteria are primarily responsible for the mineralization of organic matter and the recycling of plant nutrients. The rate of mineralization controls to a large degree the rate of primary productivity and ultimately the amount of food which can be harvested from the sea. We still have limited knowledge concerning this mineralization process. A priori the rate of mineralization must be related to the number of bacteria present in the sea.

To understand the role of bacteria in oceanic environments the concentration and distribution of these microorganisms must be determined. The number of bacteria in a sample is usually determined using plating and direct counting techniques. Using plating techniques, less than one percent of the bacteria present are detected. The direct counting technique is far from satisfactory for many of the bacteria in the ocean grow in clumps and on suspended large particles. Moreover

many of the small suspended particles in the ocean fall in the same size range as bacteria so distinguishing detrital material from bacteria becomes difficult if not impossible.

Because of the limitations of present techniques little knowledge exists concerning the distribution and concentration of bacteria in oceanic environments. We do not know for example where in the ocean most of the mineralization of organic matter occurs. Does most of the mineralization in deep oceanic waters take place in the euphotic zone, or is this process distributed throughout the water column? In shallow waters does most of the mineralization take place in the sediment or within the water column? What percent do bacteria comprise of the total biomass in oceanic environments? These are a few of the questions what we are attempting to answer.

To answer the above and additional questions considerable time was spent during this funding period working on a new method, the Limulus lysate test, to determine bacterial biomass in oceanic environments. Combining this new method with the ATP method we can determine the total biomass and what percent the bacteria represent of the total biomass.

Both laboratory and field studies have been conducted using the above techniques. The majority of our laboratory time has been spent improving the sensitivity of the test and working out adequate extraction procedures. We have employed these techniques on two cruises and are now preparing to go on a third cruise in May of 1974. On this latter cruise we plan to study mineralization in an upwelling area off the west coast of Africa.

We are developing the Limulus lysate method to determine the bacterial biomass distribution. Limulus lysate is an extract of blood cells (amebocytes) obtained from the horseshoe crab (Limulus polyphemus). This method, which is specific for detecting Gram-negative lipopolysaccharides, was originally described by Levin and Bang (1964). Lipopolysaccharides (LPS) are components of the outer cell wall layer of Gram-negative bacteria and comprise approximately ten percent of the dry weight of the bacterium. The Limulus test is so sensitive that it can detect picogram quantities of LPS or as few as 100 bacteria/ml.

This test has not previously been used to study bacteria in water. However the majority of bacteria in seawater are Gram-negative, and we reasoned that if we could measure the LPS content of seawater we would have a fairly accurate estimate of the total number of bacteria present. On this assumption we proceeded to develop this method for use in field studies to detail the distribution and concentration of bacteria in oceanic environments. This same method could be used with equal facility to study bacteria in bodies of freshwater.

Laboratory studies: The proteins of the Limulus lysate form a clot or precipitate in the presence of minute quantities of LPS. Of the twenty or more proteins that exist in the lysate only two interact with LPS. One of the proteins is an enzyme which is activated by LPS. Once activated the enzyme triggers another protein to polymerize forming a clot and/or precipitate.

The test may be read by two methods. In one method 0.1 ml of the lysate is mixed with 0.1 ml of the test solution and then incubated at 37 C for one hour. If LPS is present, a clot will be formed after this time period. To determine the concentration of LPS two-fold serial dilutions are made of a standard endotoxin solution. These are mixed with the lysate and also incubated at 37 C as previously indicated. Using this method it is possible to determine the minimum concentration of LPS which causes the lysate to clot.

In the second method the lysate is diluted five fold. The sample to be tested (0.1 ml) is added to 0.9 ml of the diluted lysate and incubated at 37 C for one hour and then read at 360 nm in a spectrophotometer. This reading is compared to a standard curve prepared by measuring various concentrations of LPS in a similar manner. With this method 6 pg/ml of LPS can be detected. Most bacteria, the size of E. coli, contain 10 fg LPS/cell. Therefore we can detect as few as 600 bacteria. This test is considerably more sensitive than the ATP test which requires the presence of 5×10^4 bacteria in order to obtain a measurement.

The initial steps in the preparation of Limulus lysate in our laboratory paralleled those described by Levin and Bang (1964) and more recently by Young et al. (1972). A 13 gauge needle was inserted into the crab's cardiac chamber by way of the dorsal junction of the cephalothorax. The hemolymph was allowed to flow directly into a pyrogen-free siliconized 250 ml centrifuge bottle containing 125 ml

of 0.125 percent N-ethyl maleimide in 3 percent sodium chloride adjusted to pH 7.4 with tris (hydroxymethyl) aminomethane buffer and warmed to 37 C before use. The amebocytes were sedimented by low-speed centrifugation (2000 RPM) for six minutes. The blue supernatant fluid was discarded and the packed amebocytes were transferred to a 50 ml siliconized glass centrifuge tube. The amebocytes were washed twice with pyrogen-free 3 percent sodium chloride to remove the N-ethyl maleimide. After washing, the cells were resuspended in pyrogen-free distilled water at a 1:3 ratio of packed cells to water and this suspension was shaken overnight at 5 C. After approximately a 12 hour extraction period at 5 C most of the amebocytes were ruptured releasing their proteins into the suspending medium. The ruptured amebocytes were pelleted by centrifugation (3000 X g) and the activity of the supernatant was determined by titering it with a known concentration of endotoxin. The endotoxin standards used were prepared by phenol extraction of intact cells and included Escherichia coli (prepared by Difco) and Klebsiella pneumoniae (prepared by the Food and Drug Administration).

Limulus lysate was prepared by most other investigators using only the above method. Using this procedure in the summer of 1972 we produced lysate which formed a clot with 1 ng/ml of endotoxin. However, in the summer of 1973 only ten percent of the lysate prepared would clot with 1 ng/ml of endotoxin. During the winter months of 1973-1974 the lysate prepared would not clot even with 100 ng/ml of

endotoxin. Other investigators experienced similar variability in the sensitivity of the lysate. This variability seemed to be related to the time of the year the lysate was prepared and to the geographic locale from which the crabs were collected.

For the Limulus test to be a useful biological tool this variability in the sensitivity of the lysate must be eliminated. We speculated that an inhibitor in the horseshoe crab blood controlled the sensitivity of the lysate. Various solvents were used in an effort to remove this theoretical inhibitor. We found that several organic solvents removed the inhibitor but the best results were obtained with chloroform. Equal parts of chloroform and lysate were mixed and shaken on a rotary shaker for one hour at 5 C and then centrifuged at 3000 X g. A large precipitate was always present in the chloroform phase which was discarded and the aqueous phase was saved. After chloroform treatment most batches of lysate formed solid gels with concentrations of endotoxins ranging from 0.2-0.6 ng/ml of endotoxin (Klebsiella pneumoniae).

When lysate was prepared by the method of Young et al. (1972), monovalent cations frequently decreased the sensitivity of the lysate. Thus the sensitivity of the test was decreased when seawater, saline or body fluids were assayed. We suspected that divalent ions were required for clot formation and that an excess of monovalent cations would attach to the sites normally occupied by divalent cations suppressing clot formation. This hypothesis seemed to be verified for when we added 0.02 M CaCl_2 to the lysate, sodium and other monovalent

cations increased the sensitivity of the lysate. The lysate prepared in this manner can now be used to assay the concentration of LPS in seawater.

Addition of CaCl_2 or MgCl_2 to the chloroform treated lysate yielded a product which normally clotted with 0.02 ng/ml of K. pneumoniae endotoxin and with 6 pg/ml of E. coli endotoxin. This method allows us to routinely prepare lysate with uniform activity. The sensitivity of this lysate is a hundred-fold greater than found using other preparative techniques.

Previously the lysate was stored frozen. Some loss of activity was noted when the lysate was frozen and stored at -20 C but no loss of activity at -76 C. We developed techniques to freeze-dry the lysate and in this state it remains stable at 37 C for a period of more than two months. When the lysate is kept at -76 C in the dry state, we believe it will retain its activity indefinitely.

We recognized that if the Limulus lysate test was to be fully utilized, the biochemical events which occur in this test must be understood. Considerable time has been spent in the laboratory carrying out biochemical studies on the Limulus lysate.

Young et al. (1972) who were the first to initiate biochemical studies on the lysate, fractionated the lysate on Sephadex G-50 and obtained three major peaks. One of these peaks contained the enzyme and another the clotting protein. Using Sephadex G-200 we obtained

four major peaks from the lysate and like Young et al. were able to separate the enzyme and the clotting protein through column fractionation.

Isolation and characterization of the clotting protein have been carried out by Solum (1970, 1973). He showed that this protein would polymerize and clot if treated with trypsin but clot formation did not occur if a trypsin inhibitor was included in the reaction mixture. In our laboratory we have verified these results. These studies suggest that the lysate enzyme, when activated by endotoxin acts in a manner similar to trypsin. Since trypsin inhibitor has no effect on the activated lysate enzyme, this enzyme is not trypsin per se.

Our main biochemical emphasis has not been on the clotting protein although we did partially purify this protein by heating the lysate to 80 C for 15 minutes. This treatment denatured the enzyme and many other proteins. After heat treatment the number of bands on acrylamide gel was reduced to five.

In an effort to characterize the enzyme we dialyzed the lysate against distilled water in a collodion bag which retained all proteins with a molecular weight greater than 70,000. The retentate, now free of the clottable protein, was adjusted to pH 6.8 and placed on a DEAE cellulose column equilibrated with 0.02 M NH_4Ac and washed stepwise with 50 ml of 0.02, 0.05, 0.1, 0.2 and 0.5 M NH_4Ac buffers. The enzyme was eluted off the column by the 0.1 M wash.

When the semi-purified clotting protein was mixed with the enzyme fraction, a clot was not formed unless endotoxin was added. The two semi-purified protein fractions displayed activity equal to that of intact lysate. Four distinct bands were formed when the enzyme fraction was subjected to acrylamide disc gel electrophoresis and stained with a protein stain. A similar migration pattern was found using a SDS acrylamide gel. When these bands were eluted from the acrylamide disc gel and mixed with the semi-purified protein, a clot was formed without the addition of endotoxin. Since there is no way to free the acrylamide gel of endotoxins the enzyme was probably activated by contaminating endotoxin. The migration pattern on the SDS gel indicated that the enzyme had a subunit molecular weight of 28,000 to 35,000.

The purification of activated enzyme fractions was easier than non-activated fractions. To prepare an activated fraction, endotoxins were mixed with intact lysate and allowed to clot. The clot was centrifuged and the clotted protein was concentrated in the pellet while the activated enzyme remained in the supernatant. This supernatant was fractionated on a G-75 Sephadex column and the peak containing the enzyme was placed on an acrylamide disc gel. Again four distinct bands were formed and when these bands were eluted off the gel and mixed with the semipurified clotting protein a clot was formed.

Even though four bands were obtained on the acrylamide disc gel we believe we are dealing with a single protein that has been broken

down to subunits of four different molecular weights. Each subunit is capable of catalyzing the polymerization of the clotting protein.

We still have little information concerning the inhibitor which is removed by chloroform extraction. We do not know how this inhibitor functions in vivo, why the concentration of the inhibitor is apparently greater in the winter than in the summer, or the chemical characteristics of this inhibitor. To date we have not studied the mode of action of the inhibitor.

Chloroform and non-chloroform treated lysate yielded an equal number of bands with similar electrophoretic migration patterns when run on an acrylamide disc gel and stained with a protein stain. However, if stained with a stain specific for lipoproteins, Sudan Black B, a single band was found in the non-chloroform treated lysate and this band was absent in the chloroform treated material. This evidence suggested that the inhibitor was probably a lipoprotein.

The time it takes for lysate to clot is contingent upon the concentration of the endotoxin. A high concentration of endotoxin will clot the lysate within 5-10 minutes, but it might take 45-60 minutes for clot formation when lesser concentrations of endotoxins are used. Once clot formation has been initiated it is critical not to shake or move the tube in which the reaction is occurring. The clot is easily disrupted and once disrupted will not reform. Recently in collaborative studies with Dr. Lewis Thomas it was shown that an inhibitor is formed or released during the clotting reaction which prevents the broken clot from reaggregating.

During clot formation the activated enzyme can be shown to remain in the supernatant and all of the LPS can be found within the clot. If the clot is resuspended and then reacted with fresh *Limulus* lysate, clotting does not occur because of the presence of the inhibitor formed during the initial clot formation. This inhibitor is heat labile and if the clot is heated to 60 C for five minutes the inhibitor is destroyed and the LPS present in the gel will stimulate clot formation when added to fresh lysate. If the original clot is separated from the supernatant and acidified, the clot dissolves. The clot will reform immediately when this reaction mixture is neutralized and this procedure can be repeated a number of times. The amount of inhibitor formed is proportional to the concentration of LPS used to stimulate clot formation. The greater the endotoxin concentration the greater the amount of inhibitor formed.

To understand the biochemical mechanisms involved in the interactions of the *Limulus* proteins and LPS one must also have a knowledge of the chemical structure of LPS and the biological activities induced by such compounds.

About the turn of the century it was discovered that sterile saline or distilled water contained a heat stable compound which would induce fever and other toxic symptoms when injected into humans and other animals. These heat stable compounds, now called endotoxins or pyrogens, were subsequently found to be lipopolysaccharide fragments of the outer-most cell wall layer of Gram-negative bacteria.

These endotoxins are constantly excreted by growing bacteria and are also released when these organisms die. Since Gram-negative bacteria grow in a wide range of habitats, the pyrogens are ubiquitous. They are quite resistant to microbial decomposition, are not easily destroyed by heat, and are present in or on nearly everything we eat, drink or touch. Fortunately LPS do not elicit toxic manifestations until they enter the blood stream.

It is generally agreed that all or most of the lipopolysaccharides are bound in some manner to the outer double-track layer in Gram-negative bacteria (Mergenhagen et al. 1966). Although the manner in which LPS is associated with the double-track layer is not clear, it is certain that the LPS is not responsible for the morphological integrity of the outer double-track layer. In our laboratory we removed all the LPS from the outer double-track layer and observed no alteration of the morphology of this layer. Yet the lipopolysaccharides may comprise 5-30 percent of the dry weight of a Gram-negative cell (Milner, personal communication). Working with cultures of E. coli we found that five percent of the cellular dry weight was composed of LPS. Since LPS do not stain in thin sections of bacteria, their exact location in the cell cannot be determined using normal electron microscopic procedures.

Shands (1965, 1966) using ferritin antibody conjugates to the LPS somatic antigens found that LPS extended up to 1,500 A beyond the apparent borders of the cell wall. He was also able to localize these somatic antigens on the outer surface of the plasma membrane.

Various investigators have shown that the component parts of the LPS molecule are located on the surface of the plasma membrane, but it is not clear whether the subunits are assembled on the membrane or transported to the outer cell surface before assemblage.

In our studies on E. coli we investigated the fate of LPS during growth and cell fractionation using the Limulus lysate test. During the lag phase of growth we found twice as much LPS in the medium than in the cells. During log phase the concentration of cell-bound LPS was twice that found free in the medium. During the stationary phase the concentration of the cell-bound LPS remained unchanged while the free LPS concentration increased and was twice that found associated with the cells. These studies indicate that LPS is being constantly produced and a significant fraction of it is being excreted into the growth medium.

The Limulus lysate test was also used to trace the fate of E. coli LPS in cell fractionation studies. Cells were ruptured with a French Press and the various components separated by differential centrifugation. Prior to rupture of the cells the concentration of the LPS, as determined by the Limulus test, was measured. When these cells were suspended in Tris, the lysate test indicated that they contained a total of 50,000 ng of LPS. After these cells were washed and resuspended in Tris - EDTA (disodium ethylenediamine tetraacetate), the lysate test showed the presence of 1,689,000 ng of LPS and over 90 percent of the LPS was attached to bacterial cells. Levy and Leive

(1970) reported that a considerable fraction of the LPS was released from cells when they were treated with EDTA. This did not seem to be the case in our studies. The fact that higher titers were found in EDTA treated cells suggests that more reactive sites were exposed or that inhibiting cations were removed.

After rupture of the cells, 1,100,000 ng of LPS were detected in the cell free homogenate. A total of 40,000 ng of LPS were removed by a 3,000 X g centrifugation and another 100,000 ng by centrifuging at 10,000 X g. Electron microscopic examination revealed that most of the cell walls were concentrated in the 10,000 X g pellet. It was surprising to find that only a small fraction of the remaining LPS appeared to be localized in this pellet. When this pellet was placed on a sucrose density gradient 440,000 ng or about 40 percent of the LPS originally present in the cell-free homogenate was found in a single band. Since the LPS concentration appeared to increase upon density gradient centrifugation, it appears that the sucrose changed the physical state of LPS permitting higher titers to be obtained.

The 10,000 X g supernatant, containing 320,000 ng of LPS, was subsequently centrifuged at 144,000 X g for an hour. This pellet contained 240,000 ng of LPS and the supernatant 93,000 ng. When this pellet was placed on a density gradient, two main bands were formed. The band containing primarily membranous vesicles was contaminated with 55,000 ng of LPS while the band which was composed of cell wall fragments contained 430,000 ng of LPS. Again since a higher titer of

LPS was found after density gradient centrifugation than before, we suspect that sucrose caused an alteration of the LPS molecule exposing more active sites.

These studies, while still preliminary, do provide some useful information. They indicate that most of the LPS remained firmly attached to the cell wall fragments. Seventy-five percent of the LPS from the various fractions was recovered in bands between 46-51 percent sucrose. About 13 percent of the LPS was released and not attached to cell walls or membrane fragments. These studies also indicate that the LPS detected by the Limulus lysate depends upon the physical state of the LPS which can be altered by sucrose and EDTA. It does appear that the Limulus test can be a useful tool to judge the separation of cell wall and membrane components in structure-function studies.

No previous evidence has been presented to indicate that LPS is ever organized in a molecular array in the cell wall. We have preliminary evidence to suggest LPS is organized in Nitrosococcus oceanus (a marine nitrifying bacteria) in 40 Å subunits arranged in a rectangular manner (Fig. 1, 2) (Watson and Remsen, 1970). Treatment of intact cells with SDS followed by treatment with RNase, DNase and lysozyme yielded a cell fraction composed of bag-like structures covered with the 40 Å subunit. The 40 Å subunits are solubilized with hot DMSO leaving only a backing layer. The solubilized material is composed of 30 percent protein and 30 percent LPS. No LPS was detected in

Figure 1

An electron micrograph of a freeze-etch preparation showing cell envelope layers of Nitrosococcus oceanus. The cell wall is composed of the W-2 layer having 50A subunits and the W-3 layer with 40A subunits. PM is the plasma membrane. LPS is localized in the W-3 layer. X190,000

Figure 2

An electron micrograph of a negatively stained preparation of the W-3 layer shown in Figure 1. X 103,000

this fraction with the Limulus lysate test until it was extracted with phenol. The fact that the LPS could be detected after phenol extraction suggests that in some cases LPS when tightly bound to a protein will not react with the Limulus lysate to form a clot. As our other studies suggested, this study also indicated that the physical state of the LPS controls the degree with which LPS will react with the Limulus lysate. These studies further suggest that LPS can be arranged in a highly organized manner in some Gram-negative bacteria.

It has also been generally accepted that all Gram-negative bacteria have LPS but this may not be the case. For example we have not been able to detect LPS in Nitrobacter winogradskyi either in intact cells or in cell-free fractions. Also we failed to find LPS in Halobacterium halobium. If LPS is present in such organisms, it is probably tightly bound to proteins which prevents it from reacting with the Limulus lysate. Future investigations should resolve the question whether all Gram-negative bacteria have LPS and how proteins tightly bound to the LPS prevent it from activating the lysate enzyme.

We have emphasized several times that the physical state of the LPS must control the degree of interaction that occurs when it is mixed with endotoxin. To understand the mechanism of action of the Limulus lysate test a comprehensive knowledge of the LPS structure is required.

The LPS of Gram-negative bacteria are polymers having a molecular weight of several million (Ribi et al., 1962). These polymers are formed by association of subunits having molecular weights of 118,000 to 10,000 following the bacterial species and the methods of isolation and determination (McIntire et al., 1969; Olins and Warner, 1967; Hannecart-Pokorni et al., 1970a,b; 1973; Ribi et al., 1966).

When examined with an electron microscope, phenol extracted LPS appears as long flexible structures of variable lengths and almost equal diameter. LPS from mutant strains may have a less regular diameter and usually appear as thick sticks. These high molecular weight polymers can be sedimented by high-speed centrifugation (100,000 X g).

How LPS molecules are arranged in these aggregates has not been clearly explained. Some of the proposed models include (1) a micellar arrangement (Ribi et al., 1966), (2) a trilaminar structure (Shands et al., 1967; Shands and Graham, 1969), (3) a bimolecular leaflet (Rothfield and Horne, 1967).

The subunits of the aggregates of LPS are held together by hydrophobic bonds due to the presence of lipids as well as by the chelating activity of calcium and magnesium (Hannecart-Pokorni et al., 1973). The LPS of Shigella flexneri (Hannecart-Pokorni et al., 1970a,b) was shown to be composed of 1-S subunits which could be dissociated from the aggregate by treatment with deoxycholate, chelating agents and lipid solvents. The dissociated subunits could be reassociated

once the dissociating reagent was removed. Frequently the treatment with some reagents (i.e., citrate buffer) resulted in a irreversible disaggregation.

If the Limulus test is to be fully utilized, it is essential to determine if the lysate reacts only with aggregates of LPS, or whether subunits of LPS can activate the lysate enzyme. Preliminary studies in our laboratory suggest that phenol extracted LPS treated with EDTA either irreversibly breaks down to subunits or alters the active site. Even after removal of the EDTA from the treated LPS, the phenol extracted LPS did not trigger clot formation in the lysate.

While EDTA inactivates phenol-extracted LPS, quite the opposite occurs when native LPS is treated with EDTA. As previously explained, EDTA appears to increase the number of active sites responsible for the reaction with Limulus lysate. Since phenol-extracted LPS is free of protein, its chemical nature must be dissimilar from native LPS as is its biological activity.

The LPS polymer includes the "Lipid A" moiety, the inner core region, which is basically composed of glucose, heptose, and 3-deoxyoctulosonic acid (KDO) and the outer-core region which provides a linkage site for the ultimate attachment of the O-antigen polysaccharide. The reader is referred to Osborn (1969) for a more complete review of the biochemistry of the polysaccharide moiety.

Most investigators believe that the "Lipid A" moiety is primarily responsible for the biological activity of LPS. This fraction contains

glucosamine, and fatty acids comprised primarily of myristic and β -hydroxy-myristic acids. The latter is a specific component of "Lipid A" and it is present in amide linkages to the amino groups of the glucosamine residues. KDO is believed to be in ketosidic linkage to "Lipid A", however, the site of attachment remains unknown (Osborn, 1969).

It is not known whether the intact LPS molecule is required to induce all of the biological reactions, or whether a subunit of the LPS, such as "Lipid A" is entirely responsible for the biological activity of this molecule. We do not know if one active site on the LPS molecule is solely responsible for the biological activity, or whether several active sites exist each of which may elicit a specific biological reaction.

There is still debate concerning which portion of the LPS molecule is responsible for the endotoxic properties. Ribi et al. (1959, 1961) obtained a highly toxic endotoxin by mild extraction procedure. This preparation had a low fatty acid content and thus Ribi argues that lipids are not primarily responsible for the toxic properties of endotoxins.

Most workers, however, contend that the "Lipid A" part of the molecule is primarily responsible for the endotoxic activities manifested by LPS. Several workers (Westphal and Luderitz, 1954; Howard et al., 1957; Kasai et al., 1961; Mihich et al., 1961; Neter et al., 1960) showed that the "Lipid A" moiety maintained 5-10 percent of the

original endotoxic activity. Westphal and co-workers (Westphal, 1960; Westphal and Luderitz, 1954; Westphal et al., 1958) were the first to call attention to the significance of the "Lipid A" part of the LPS molecule.

Previously most studies indicated that the intact lipopolysaccharide had greater biological activity than the "Lipid A" fraction alone. However, in the past it was difficult to measure the full biological activity of "Lipid A" because of its relatively insoluble property in aqueous solutions. Recently new methods have been devised to solubilize the "Lipid A". Using solubilized "Lipid A" Thomas (personal communication) found that the Schwartzman activity of "Lipid A" was much stronger than when intact LPS was used. Previously it was reported (Kasai et al., 1961) that the Schwartzman activity of the "Lipid A" fraction per se was quite weak and less than one-hundredth of intact LPS. Thomas (personal communication) recently found that clot formation in the lysate can be triggered by a solubilized "Lipid A" fraction. In a collaborative study with Dr. Dennis Watson we compared the titer of a "Lipid A" fraction derived from a mutant Salmonella minnesota (Strain 595) to the intact LPS extracted from this organism. The titers obtained with both fractions were identical.

All of the recent evidence seems to suggest that the "Lipid A" moiety is primarily responsible for the biological activity of the LPS. Even if future experimental data verifies this fact it still leaves the question unanswered of how "Lipid A" triggers such a wide variety of biological reactions. What are the active sites on the "Lipid A"

molecule? Is the same active site responsible for triggering all of these biological reactions? Can for example the "Lipid A" be modified so it will exhibit anti-tumor activity but not produce unwanted toxic reactions? Does the association of the "Lipid A" fraction with polysaccharides or proteins alter its biological activity? Why does LPS have such a great affinity for and more destructive effects on primitive cells than on other cells? Is there any relationship between "Lipid A" reaction with the Limulus lysate and with neoplastic cells? These are only a few of the questions that need to be answered about the biological activity of "Lipid A" and intact LPS.

If specific active sites on the "Lipid A" or some other fraction of the LPS molecule are responsible for evoking many different biochemical events, then an inhibitor effecting one biological system may effect others as well. A clue to what types of compounds may inhibit or decrease the biological activity of LPS has been provided by the work of Springer et al. (1966). These investigators examined the types of compounds which inhibited or suppressed the red cell-lipopolysaccharide interaction. The types of compounds which inhibited the attachment of LPS included: water-soluble lipid structures, small molecular size water-soluble aliphatic carbonyl compounds possessing adjoining hydroxyl or ethylene groups, and basic peptides including globin.

Similar type compounds may interfere with the clotting of Limulus lysate when exposed to LPS. Lipoproteins interfered with the red cell-lipopolysaccharide interaction and appeared to block the activation

of the enzyme by LPS in the Limulus lysate reaction. A lipoprotein also exists in human plasma which interferes with the Limulus test. It is suspected that lipoproteins play an active role in vivo both in horseshoe crabs and humans. In horseshoe crabs the lipoproteins must control the clotting mechanism and prevent clot formation in the crab. In human blood lipoproteins may inactivate endotoxins and provide protection against the presence of small amounts of endotoxin in the blood.

These laboratory studies have provided background information for our field studies. In summary they have shown that the Limulus lysate test is specific for Gram-negative lipopolysaccharides and detects picogram quantities of such compounds. All evidence suggests that only the "Lipid A" moiety of the LPS is being detected with this test. We know certain compounds exist which interfere with this test and the detection of every "Lipid A" molecule is not yet possible. Often the "Lipid A" moiety appears to be masked by proteins and other molecules which possibly interfere with the active sites on the "Lipid A" moiety. We are apparently unmasking these active sites on the natural LPS by treatment with compounds such as EDTA and detergents such as sodium laurylsulfate (SDS). Whether we are unmasking all active sites by treatment with these compounds must still be experimentally verified. While we have learned a great deal about the biochemical mechanisms involved in this sensitive test, some factors remain unknown. However we feel that we have sufficient understanding

to use this test profitably in field studies and we have spent considerable time in the development and application of this test in ocean studies. It is important to continue our laboratory studies for information obtained will provide the understanding needed for the interpretation of the findings of field studies.

Field studies: The Limulus test measures only the total LPS (or a percent of the total) present in any water or sediment sample. This LPS exists in live bacteria, dead bacteria and in a free state not bound to any microorganism. To date a method to distinguish the LPS derived from dead bacteria from that derived from living bacteria has not been devised. Even the separation of bound from free LPS is a difficult task. This can be accomplished by high speed centrifugation but this technique does not lend itself to shipboard use. Laboratory studies indicate that approximately half of the LPS in seawater is in the bound state.

Originally it was believed that filtration would easily separate bound from cellular LPS. We could show that phenol extracted LPS would pass through and would not be absorbed to a filter. However when we passed seawater through the filter, we could not recover all of the LPS originally measured in the raw seawater. Usually about 25 percent of the LPS in the seawater was found in the filtrate and 10-50 percent on the filter. The reason or reasons why we could not recover all of the LPS retained on the filter is still not clear. Possibly some of the cell wall fragments containing LPS became physically entrapped within the membrane. Since extraction procedures do not

completely solubilize these fragments, the LPS never becomes freed from the filter. Our experiments have revealed that at least 25 percent of the LPS present in seawater is in fragments less than 0.2 μ in diameter.

The above experiments do not necessarily indicate that the remaining 75 percent of the LPS is bound to bacterial cells. When seawater is centrifuged at 5,000 X g, 50 percent of the LPS is pelleted and when centrifuged at 10,000 X g 75 percent of the LPS is concentrated in the pellet. These experiments suggest that at least 25 percent of the LPS is not bound to cells since most cells will be pelleted at speeds of 5,000-10,000 X g. Normally 5,000 X g will pellet intact cells but not large cell fragments and 10,000 X g will pellet both cells and large cell fragments. Thus it appears that about 50 percent of the LPS is attached to cells, 25 percent is attached to large cell wall fragments and the remaining 25 percent are in an extremely small state. These percentages will change from sample to sample, but experiments of this kind provide a rough idea of the LPS distribution based on various size particles. They also indicate that if we estimate the total number of bacteria present in non-filtered water by the Limulus technique the estimate should not be off by more than 50 percent.

In spite of the technical difficulties encountered, we have used the Limulus test on two cruises. In the upper 200 meters in open ocean waters a sufficient amount of LPS exists permitting the

elimination of filtration or other concentrating techniques. We collected samples every 10 meters from 0-200 meters at 45 stations on the cruises between Woods Hole and Bermuda and Lisbon and the Azores. The concentration of LPS found in these samples suggest that there are 10^3 - 10^5 bacteria/ml in the upper 200 meters and that a maximum concentration of bacteria exists just below the euphotic zone.

The concentration of LPS was too low below the depth of 1000 meters to determine the LPS concentration without filtration. One liter samples from these depths were filtered through a 0.2μ Millipore filter and the LPS was extracted from these filters. Assuming that only 10 percent of the LPS originally present in these samples was extracted from the filters and applying the appropriate correction, the LPS concentration of extracted LPS indicated that not more than 10 bacteria/ml existed in the deep waters of the ocean. This indicates that over 99 percent of the mineralization of organics in the deep oceans must occur within the upper 200 meters of the water column. The waters below a depth of 1000 meters must be considered as a microbial desert where little if any microbiological activity occurs.

Since the samples collected from deep waters would be essentially free of organisms other than bacteria, the total biomass in these samples should be comprised of bacteria. If this assumption holds, a fairly good agreement between total biomass measurements made by the ATP method and the LPS biomass measurement should be evident.

Using the ATP method in conjunction with the Limulus assay should also permit one to determine what percent of the biomass is comprised of bacteria in the euphotic zone.

An ATP method similar to the one outlined by Holm-Hansen and Booth (1966) was employed for total biomass measurements. For these measurements samples (200-1000 ml) of seawater were first filtered through a 0.2 μ Millipore filter. Before the filter dried it was placed into 5 ml of boiling Tris buffer for five minutes and then immediately cooled on ice. After cooling 10 μ l of the extracted ATP solution was injected into a DuPont Luminescence Biometer which could detect as little as 5×10^4 fg of ATP. Laboratory studies have shown that each bacterium, the size of E. coli, usually contained 0.5-1.0 fg of ATP.

In samples from below 1000 meters a good agreement was obtained between the number of bacteria calculated to be present using the ATP and the LPS methods suggesting that most of the LPS being measured was in the living state. The concentration of ATP in deep samples also indicated there were 10 or fewer bacteria/ml.

The ATP concentrations measured in samples from the upper 200 meters of the water column suggest that bacteria comprise only 10 percent of the biomass at these depths. However respiration studies have indicated that the bacteria in the upper 200 meters of the water column can account for up to 50 percent of the total oxygen consumption in these waters. These facts demonstrate that bacteria play an important

role in the mineralization of organics in the upper 200 meters of the water column.

Thus far we haven't used the ATP and LPS methods to study the concentration and distribution of bacteria in offshore marine sediments. At present we are working on techniques for measuring LPS and ATP in local water sediments and expect to apply these techniques on a cruise off the coast of Africa in May 1974. To measure the LPS in sediments one gram is suspended in 0.1 percent SDS and boiled for five minutes and then centrifuged for five minutes at 3000 X g to remove the sediments. Ten fold dilutions are made of the supernatant. Each supernatant dilution (0.1 ml) is mixed with 0.2 ml lysate, diluted to 5 ml and incubated at 37 C for one hour and read in a spectrophotometer at 360 nm. A standard curve is similarly run using appropriate concentrations of LPS. The concentration of LPS in the diluted extracts of the sediment samples can be obtained from the standard curve.

ATP was extracted from sediment samples following basically the techniques of Lee et al. (1971). Wet sediment samples (0.2 gm) were mixed with 5 ml of 0.6 N H_2SO_4 and blended in a Virtis tissue blender at 60,000rpm for 30 seconds at 5 C. After homogenizing the sample incubated on ice for 20 minutes, centrifuged and the supernatant saved. Two ml of the supernatant were mixed with 5 ml of Amberlite IR-120 resin to remove metals. After a 3 minute treatment with the resin the solution was decanted, the resin was washed twice, and all decanted solutions combined. The pH of the solution was adjusted to

7.8 with NaOH and made up to a final volume of 10 ml. Ten fold dilutions of this solution were made and 10 μ l of each dilution measured in the ATP biometer.

ATP was also extracted from sediments by another method suggested to us by Dr. Wiebe (unpublished information). In this technique 1 gm of sediment is added to 9 ml of 0.1 M NaHCO_3 and boiled for 30 seconds. After boiling 0.1 ml of the NaHCO_3 solution was added to 0.02 M Tris at pH 7.75 on ice. The Tris solution was injected as rapidly as possible into the Biometer.

Using the acid extraction procedures 5×10^7 fg/gm of sediment ATP were found; while using the NaHCO_3 method $1-7 \times 10^8$ fg/gm of sediment ATP were measured. If we assume that most of ATP measured came from bacteria, and that each bacterium contained 1 fg of ATP, we estimate that there would be $1-7 \times 10^8$ bacteria per gram of sediment.

One gram of these same sediments contained 40 μ g of LPS. If all of this LPS existed in living bacteria we estimate that there would be 2×10^9 bacteria per gram. We anticipate that not more than 25 percent of this LPS is associated with living bacteria. If this assumption is true, then based on the LPS method, we estimate that there are 5×10^8 bacteria per gram of sediment which agrees quite closely to the method obtained using the ATP method using NaHCO_3 extraction.

Assuming that in shelf water a similar distribution and concentration of bacteria in sediments and the water column occurs and assuming a water depth of 100 meters it can be estimated that under one square

meter of sea surface 10^{13} bacteria are in the water column and 10^{13} bacteria are in the upper 10 cm of the sediment. Based on this distribution of bacteria, it might be argued that 50 percent of mineralization occurs within the water column and the remainder in the sediments. However the respiration rates are not governed entirely by the number of bacteria present in each environment.

To study the correlation between cell number and rates of oxidation, C^{14} labelled acetate ($1 \mu\text{m/L}$) was added to samples of seawater and sediment and incubated for four hours. After incubation the released $C^{14}O_2$ was counted in a scintillation counter. Similar experiments were also conducted with a C^{14} urea. With either substrate on a equal volume basis the oxidation rates in sediments was ten times that found in the water. As previously indicated the number of bacteria in sediments (on an equal volume basis) is 1000 times that in the water column. Therefore one might expect that the respiration rate in the sediments would exceed that in the water column (equal volume basis) by 1000 fold but this does not seem to be true. Instead the respiration on a per cell basis appears to be ten times greater in the water than in the sediments. This would suggest that only ten percent of the mineralization in shallow waters (100 m depth) takes place in the sediment.

If 100 grams of carbon are fixed/sq. meter/year in coastal areas, and if bacteria are responsible for the oxidation of half of this amount, then we estimate only five grams of carbon is oxidized per sq.

meter per year in the sediments. In the local waters less than 10 meters deep about 200 gm of carbon are fixed per year. Because the waters are shallow there are ten times more bacteria under one square meter in the sediment than in the water column. Thus we estimate 50 percent of the mineralization occurs within the sediment. If this estimate is correct 100 gm/sq meter/year of organic matter would be oxidized in local sediments. Based on our calculation of the number of bacteria present, the bacterial biomass under one square meter would represent five grams of organic carbon. In contrast the macrofauna would contain 50 grams of carbon. Respiration rates measured by Dr. Kenneth Smith suggest that bacteria are responsible for 50 percent of the oxygen consumed in the sediments. Thus bacteria would utilize about 50 grams of organic carbon per year. If we assume that 50 percent of the organic carbon utilized by the bacteria is incorporated as cellular material and that the death rate of these bacteria equals their division rate then it appears that the bacteria would divide only a few times per year.

These facts suggest that the bacterial populations in the sediments do not have the ability to change rapidly in response to substrate concentration. They have adapted to the environment and are slow growing organisms and for this reason they can survive under the conditions found in the sediment. If this logic has merit one would not expect that sewage or other organics added to sediments would be decomposed rapidly. This fact cautions against dumping sewage and

other organics in even inshore waters.

Our experimental data thus far obtained is too sketchy to permit any broad generalizations concerning the rates and sites of mineralization. The views expressed above can only serve as a working hypothesis for future studies. If these expressed ideas are viewed in this light then they can serve a useful purpose.

Medical studies with the Limulus lysate: The Limulus lysate may serve as a useful tool in several scientific disciplines. Since the original description of the lysate method for the detection of endotoxins (Levin and Bang, 1964) it has been employed for the detection of endotoxemia (Levin et al., 1972; Levin, 1973), bacteriuria (Jorgensen et al., 1973) and for the diagnosis of Gram-negative spinal meningitis (Nachum et al., 1973). We have entered into a collaborative study with the latter group from the Childrens Hospital in Los Angeles and also expanded this study to include clinicians at the Childrens Hospital in Washington, D.C. During the last year it has been used to help diagnose the etiological agent causing spinal meningitis in over 500 children. To date a false positive or a false negative result has never been obtained. Recently we have initiated another collaborative study with Dr. Dass from the Children's Hospital in Boston. In this study we will study the incidence and the severity of endotoxemia in children with severe cases of diarrhea. When a child has a severe case of diarrhea much of the intestinal mucosa is destroyed and endotoxins seep into the blood stream and death often results.

We have also carried out collaborative studies with the M.D. Anderson Tumor Clinic in Houston, Texas on detection of endotoxemia in cancer patients. Frequently the permeability of the intestine is decreased in cancer patients and endotoxemia results causing spiking fevers in the patients. The Limulus lysate has been used with these patients to determine the degree of endotoxemia.

The Limulus lysate should also be a useful tool in patients who have undergone severe burns. Most of the deaths in burn patients stem from Pseudomonas infections. When a patient is severely infected, endotoxins enter the blood stream and death results.

We believe it should also be a useful tool in studying patients who have been exposed to high concentrations of radiation. A priori one would expect the permeability barrier of the intestinal mucosa to be lowered if there is extensive radiation damage. If this occurs endotoxemia could cause death in a relatively short period of time. We do not know of anyone who has actually used the Limulus lysate test to study the incidence of endotoxemia in patients exposed to high doses of radiation.

The half-life of many radiochemicals is too short to permit their endotoxin content to be monitored by the rabbit fever test routinely used by drug firms. Since autoclaving does not destroy endotoxins special precautions must be taken to insure radiochemicals are free of such toxic compounds. The Limulus test could be profitably used to determine whether radiochemicals are free of endotoxins prior to their use with patients.

The collaborative medical studies which have been carried out in this laboratory have been supported with non-federal funds. However much of the knowledge which we used in these studies was derived from basic studies supported by A.E.C.

Studies of the nitrogen cycle: For several years the A.E.C. has supported our studies on the nitrogen cycle in the oceanic environment. Mainly these studies have dealt with the process of nitrification. During the last year we initiated studies on nitrogen fixation in the marine environment.

To balance the global nitrogen budget Hardy and Holstein (1972) estimated that 40×10^6 tons of N_2 must be fixed annually in the ocean. This estimate implies that 20-25 percent of all nitrogen fixation occurs in the ocean but there is no experimental evidence to indicate how much total N_2 is fixed, which organisms are responsible, and if most fixation occurs in inshore or offshore environments. High rates of N_2 fixation have been found in some inshore waters. For example, bacteria associated with marine angiosperms fix as much N_2 /ha as leguminous plants (Patriquin and Knowles, 1972). Blue-green algae are common inhabitants of many shallow water communities and they too must fix significant quantities of N_2 . Nitrogen fixation in inshore waters however cannot account for the amount of nitrogen fixed postulated by Hardy and Holstein. For this reason we speculated that most of the nitrogen would be fixed in offshore environments.

Only four brief reports exist concerning nitrogen fixation in open ocean environments and these deal with two genera of blue-green algae (Dugdale et al., 1961, 1964; Bunt et al., 1970; Carpenter, 1972). The amount of nitrogen fixed by these organisms appears insufficient to balance the global nitrogen budget. We thought that possibly bacteria were fixing significant quantities of nitrogen in offshore waters and thought this possibility should be examined.

Nitrogen fixing bacteria could be routinely isolated from in-shore waters. Usually their number ranged from 10-1000/ml. Using the acetylene technique we were unable to measure, on a short term basis, nitrogen fixation in raw seawater samples. On occasion we found nitrogen fixing bacteria in shelf waters, but the number present would fix an insignificant quantity on an annual basis. In offshore waters we failed to culture nitrogen fixing bacteria directly from samples of water.

We did find microorganisms associated with the pelagic alga, Sargassum natans, which fixed significant quantities of nitrogen as judged by the acetylene-reduction technique (Hardy et al., 1968). Samples of S. natans collected on a cruise in the Sargasso Sea were incubated in small serum vials in the light and dark. Microscopic examination of the surface of S. natans revealed numerous bacteria but no blue-green algae. Nitrogen fixing bacteria were easily cultured from S. natans suggesting that bacteria rather than blue-green algae were the biological agents responsible for nitrogen fixation. Other evidence also substantiated this belief.

Usually 44-55 percent more nitrogen was fixed in the light than in the dark but in one experiment more fixation was detected in the dark. Nitrogen fixation rates should have dropped precipitously in the dark if blue-green algae were responsible and this did not occur.

It is believed that bacteria growing on the surface of the algae were fixing nitrogen at the expense of the organics excreted by the algae. The concentration of these organics would govern the rates of nitrogen fixation and this was evident in our experiments. Rates of nitrogen fixation were highest in the light suggesting that S. natans excreted more organics in the light than in the dark. The highest rates of nitrogen fixation did not occur during the first three hours. Concentrations of organics were likely not optimal during this period. Rates of nitrogen fixation were also controlled by the concentration of inorganic ions. Enrichment with inorganic salts stimulated nitrogen fixation both in the light and dark. It is assumed that the enrichment with phosphate or some other ion stimulated bacterial nitrogen fixation per se.

S. natans was composed of 1.6 percent N, 33.0 percent C and 5.25 percent H and had a C/N ratio of 20.9. This ratio indicated that this alga had an acute nitrogen deficiency. We postulated that the nitrogen fixing bacteria were supplementing the nitrogen needs of this plant but this was not experimentally verified. In these experiments we showed that the nitrogen fixing bacteria could supply only 0.13 percent of the nitrogen needed for plant growth. The average rate of nitrogen fixation (1.48 mg N/ha/day) by organisms associated

with S. natans was far less than the rates reported for other marine nitrogen fixing organisms.

These studies did not reveal that bacteria were fixing significant quantities of nitrogen in the water column. Similar studies on sediments, incubated both aerobically and anaerobically, failed to show that nitrogen fixation was even occurring along the ocean floor.

Initially we had proposed to also study nitrification and denitrification in oceanic environments. Lack of time prevented us from working on these processes during this funding period.

Percentage of time devoted to project: The principal investigator has devoted approximately 50% of his time from June 30, 1973 to April 17, 1974 on this project. He expects to devote all of his time during the month of May 1974 and one half of his time in June 1974 to this project.

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