38. MURAMIC ACID AS A MEASURE OF MICROBIAL BIOMASS IN BLACK SEA SEDIMENTS

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ABSTRACT

Muramic acid, an amino acid found only in the cell wall polymers of bacteria and blue-green algae, has been detected in deeply buried sediments of the Black Sea.

INTRODUCTION

It has unfortunately taken a long time to convince microbiologists that the techniques that have produced such remarkable progress in the treatment of infectious disease are of limited usefulness when studying ecological interrelationships. Classically the offending microbe was isolated from the host, grown in pure culture, and its physiology studied and then used to produce similar pathology in a second host. Then the highly successful technique of isolation by the selective cultural methods was tried on the microbial flora of natural systems. This proved successful in the biological monitoring of fecal pollution, and in other conditions where quantitative or qualitative assessment of so-called "indicator" organisms can give needed information.

Where a microbial process such as sulfate reduction involves a population, determination of the microbial mass by classical methods is not accurate. The classical method for the quantitative determination of microbial mass has been to sample the material in question, dilute and inoculate Petri plates containing various media. The composition of the media can be varied to give selective advantage to the types of organisms of special interest. From the number and types of colonies one can estimate the number and activities of the organisms. Studies of these activities could be amplified by studies of the physiological biochemistry of the organisms after their isolation from the colonies. Determination of microbial activities in complex environments clearly cannot be deduced from the behavior of pure cultures of organisms or from the enumeration by classical dilution and plating.

1) **Plate counts versus direct microscopic counts:** From the early studies of Butkevich (1932), the number of organisms in a fresh-water column determined by direct microscopic count was 200 to 5000 times the number predicted from the plate count. Collins and Kipling (1957) found from 6 to 11,000 times as many microorganisms by direct count as by plate count in the waters of Lake Windemere. In soils, the average direct microbial count is 10^8-10^9 cells/gram dry weight, of which only 1% to 10% may be detected by plate count (Alexander, 1961). In the sea, ZoBell (1946) reported a very small proportion of the organisms seen on slides was detected by growth on plates. Jannasch and Jones (1959) conducted a detailed study of the microbial content of seawater, comparing five types of plate counts with two types of direct counts. They found that the Cholodny (1928) technique of filtering seawater and concentrating the particulates on glass slides gave about 10^4 more organisms than the plate counts in a given water sample. Concentrating the microbes from seawater with membrane filters and then suspending the filters in medium with dilutions using the most probable numbers (MPN) technique gave values 23-25 times greater than the plate counts. Not only were the plate counts of classical microbial ecology underestimated by one to five orders of magnitude, but there were spiral-shaped organisms seen in direct counts of formaldehyde-treated dilutions that were not seen in plate counts. Other studies have shown no correlation between direct and plate counts (Skinner et al., 1952). Indeed whole groups of unusual shaped organisms have been seen to grow in special flattened capillary tubes implanted in soils and aqueous sediments. Most of these organisms have never been successfully cultivated outside the environment (Perfil'ev and Gabe, 1969). These authors found ratios of direct counts in their microchamber to plate counts of 13,000:1 to 737,000:1 in fresh-water sediments.

2) **Viability of organisms detected by direct counts:** An argument has been made that the high direct counts contain a high proportion of dead organisms. Using the principle that certain vital dyes are concentrated by living cells, the proportion of metabolically active cells in a direct microbial count can be determined. Strugger (1949) found that less than 10% of the cells contained no acridine orange in soil samples. A similar figure was determined by Alfimov (1954) in studies of lake water using erythrosine as the vital stain.

Cultures, in which each individual microorganism is able to grow, increase in mass exponentially (Brock, 1971). Essentially all cells of the filamentous organism *Leucothrix mucor* incorporate ^{3}H-thymidine exponentially if growing free in laboratory cultures. When ^{3}H-thymidine incorporation into cellular DNA, followed by microradiography, was applied to *L. mucor* growing as an epiphyte on marine algae, growth was clustered to between 50% and 70% of the filaments (Brock, 1966). Even though cells were non-growing in terms of DNA synthesis, they were able to concentrate ^{3}H-glucose, showing that even though growth in nature

may be non-random or clustered, the great majority of cells are viable and metabolically active. In other studies, nearly all the microorganisms that became fixed to sterile glass slides suspended in a pond were able to divide (Bott and Brock, 1970).

In summary, a classical method of microbiology—the viable plate count—is totally inadequate to measure the microbial biomass. The excuse that the difference between direct microscopic counts (applicable only to unattached microflora) and plate counts reflected a large proportion of dead or metabolically inactive cells is clearly not true; the majority of direct examinations of living microbial assemblages indicate nearly 100% viability.

What then are the possible methods of assessing the activities of a microbial community where the association with other microorganisms and the microenvironment can be preserved?

3) **Use of universal microbial cellular constituents to determine microbial mass:** Holm-Hansen (1973) has determined that many different types of microorganisms in the water column contain a level of adenosine triphosphate (ATP) corresponding to 0.04% of the cellular organic carbon content. Using this method, he has shown that ATP levels (which are rapidly dissipated following the metabolic death of the organism) indicate that between 50 to 2000 times more microorganisms are present in the seawater column than can be determined by plate counts (Holm-Hansen and Booth, 1966).

The ATP method has been widely used with water column microflora but has encountered difficulties when used with sediments. There are two difficulties:

1) The ATP must be quantitatively extracted from the cells and not absorbed by the sediments and be dissolved in a solution that does not inhibit the luciferin-luciferase enzyme system needed to detect the nanogram quantities present in the environment (Karl and LaRock, 1975).

2) The analysis must be done rapidly, as there are changes in ATP content in bacteria in seconds when adverse environmental conditions supervene (Goldenbaum et al., 1975).

In the present studies, we have chosen a second method in which the microbial biomass, but not its activity, could be examined from the sediments. This involves the measurement of muramic acid (3-O-carboxyethyl-D-glucosamine) in the acid hydrolysates of the sediments. Muramic acid forms a part of the backbone of the bacterial cell wall. The so-called muramo peptide is a component uniquely found in all known bacteria and blue-green algal cell walls, with the exception of the mycoplasma or PPLO organisms. The muramo peptide polymer is the main supporting material of the microbial wall and gives the cell its characteristic shape. Chains of alternating residues of N-acetylglucosamine and N-acetyl muramic acid are covalently linked by amide bonds at the muramic acid by peptide bridges. This polymer apparently completely encloses the cell. Muramic acid (3-O-carboxyethyl-D-glucosamine) does not occur in other prokaryotes or eukaryotic cells. Of the 300 or so PPLO, mycoplasma or L-forms described, many are intracellular parasites of plants and animals, although they may survive in high osmotic environments like sewage. They form a minute percentage of the bacterial mass in vertebrates. One thermoplasma has been isolated from a high temperature acidic coal mine gob pile. Others have recently been detected in the sea. No estimate of the number of PPLO organisms detected on estuarine detritus or marine environments has yet been made (Razin, 1970; Smith, 1964), but their contributions to the microbial mass appear to be minimal.

Batch cultures of organisms grown to the stationary phase contain organisms of all ages in a relatively poorly controlled environment when compared with organisms grown in a nutrient-limited chemostat (Reavely and Burge, 1972). Studies of numerous species of bacteria have shown that the proportion of muramic acid in the cell wall and the ratio of muramic acid to glucosamine are essentially invariant (Reavely and Burge, 1972; Rogers and Perkins, 1968; Salton, 1960). However, differences in the sensitivity to the wall of lytic enzyme lysozyme heat-killed *Bacillus subtilis* W-23 grown in chemostats, with different limiting nutrients, have been shown (Ellwood and Tempest, 1972). On isolation of the walls after removal of the teichoic acids and analyses of the mucopeptide, the maximum variability in the ratios of glucosamine to muramic acid varied between 0.5 for sulfate-limited growth to 1.07 for NH_3-limited cells, possibly a variation by a factor of 2. However, no changes in muramic acid levels have been induced with other strains of *Bacillus subtilis* (Young, 1965). Direct analysis of laboratory-grown batch cultures showed an average of 3.44 ± 0.5 ($X \pm \sigma$) μg/mg dry weight for seven species of gram-negative bacteria, and 9.6 ± 1.9 ($X \pm \sigma$) for five species of gram-positive bacteria, with the data expressed as μg muramic acid per mg dry weight (Millar and Casida, 1970). Spores from gram-positive bacilli averaged 38 μg muramic acid/mg dry weight ± 6.2 ($X \pm \sigma$) μg/mg dry weight. The hydrolysates of these spores also contain the amino acid dipicolinic acid, an amino acid that has not yet been found in vegetative bacteria, other prokaryotes, or eukaryotes (Chapman et al., 1971). Our methodology (see below) allows for the detection of this amino acid. As yet dipicolinic acid has been undetectable in estuarine samples.

Knowing that the muramic acid content could double due to extreme environmental stess, as has been shown for one species, that both gram-positive, gram-negative cells, and spores are present in nature, and assuming they are like laboratory batch cultures, then the muramic acid content per mg dry weight of bacteria would range 6.4 ± 3.3 ($X \pm \sigma$) with extremes of 3.0 and 15 μg/mg dry weight.

BACKGROUND

Sulfate reduction to satisfy the sulfur requirements of living cells is a property of most plants and bacteria (Roy and Trudinger, 1970). These reduction reactions, used only to satisfy biosynthetic requirements, are collectively known as assimilatory sulfate reduction. The reactions involve an initial activation of the sulfate with ATP to form adenylphosphosulfate (APS), so-called active sulfate, which then reacts a second ATP to

form 3-phosphoadenyl sulfate (PAPS), where it enters biosynthetic pathways.

A second class of microorganisms utilizes sulfate as a terminal electron acceptor and forms large amounts of sulfide in the process. These are known as the dissimilatory sulfate reducers. Again, they must activate sulfate to APS, which is then reduced to sulfite and AMP. These organisms do not contain PAPS-forming kinase. The reactions by which sulfite is reduced to sulfide involve several groups of proteins of which one component contains the unique octa-carboxylic tetrahydroporphyrin prosthetic group.

Two major groups of microorganisms occupy the restricted ecological niche of dissimilatory sulfate reduction, and are most likely to be responsible for the enrichment of ^{32}S or its natural abundance in sulfur domes (Thode et al., 1954). Although the organisms have different morphology, they have similar enzymes which help define their evolutionary relationships (Peck, 1974). The most frequently found organisms are the *Desulphovibrio* organisms; these are obligately anaerobic, non-sporulating curved rods that stain negative with the gram stain. Much less frequently detected are the *Desulphotomaculum* group of organisms which are spore-forming, anaerobic, rod-shaped bacteria which stain positively with the gram stain. The gram stain is a staining technique.

The classical culture medium of the *Desulphovibrio* requires a low Eh (anaerobic conditions) (Connell and Patrick, 1968), and an alkaline *p*H (LeGall, 1973). However, sulfate reducers have been isolated from acid sediments in lakes (Ivanov and Karavaiko, 1966), and peat bogs at *p*H 3-4 (Kuster, 1969). They have been detected in saline environments (LeGall, 1973), where structures of the wall and membrane determine the ability of organic solvents to remove stains, and the distinction of positive or negative has proved a convenient mechanism for biochemical classification of bacteria (Brock, 1974).

Despite a great deal of work, these two classes of organisms appear to be the important sulfide-producing microorganisms. In the extremely protein-rich environments like rotting meat, several species of bacteria and yeasts are capable of generating hydrogen sulfide, as well as some of the volatile alkyl sulfides that give these environments their particular smells and tastes (Kadota and Ishida, 1972).

Certain characteristics of the dissimilatory sulfate-reducing bacteria have been outlined.

Syntropy, associations of the *Desulphovibrio* types of organisms with the organisms that can supply organic nutrients, are well known in nature. Tezuka has extensively studied the association of enteric organisms with the sulfate reducers in the Sumida River near Tokyo (Tezuka, 1965, 1966; Tezuka et al., 1963). Carbon sources appear to be limiting sulfate reduction in many environmental situations (Cahet, 1966). The principal organic substrates utilized by *Desulphovibrio* are the short-chain fatty acids (Postgate, 1965); the action of the syntropic organisms appears to represent the supply of these materials.

The rate of sulfate reduction is clearly dependent on the nature and amount of organic carbon substrate. Ramm and Bella (1974) have shown that the rate of sulfide reduction in the marine water column is given by the expression $ds/dt = 77$ (soluble organic carbon/[650 + soluble organic carbon]) for "fresh" soluble organic carbon in g/liter. In strip mine effluents containing 890 μg sulfate/ml at 37°C, fresh wood dust induced sulfate reduction rates of 70-80 μg/ml/day whereas more refractory wood dust residues from prior incubation supported progressively slower rates of degradation (Tuttle et al., 1969). The addition of sulfur-containing pesticides to sediments can increase the rates of sulfate reduction (Sherman et al., 1974).

The effect of sulfate concentration on the rate of reduction appears independent of sulfate ion at concentrations greater than 10 mM (960 ppm) (Kaplan and Rittenberg, 1964). However, at lower concentrations the rate of reduction appears to be first order (Nakai and Jensen, 1964), although recent studies (Martens and Berner, 1974) claim the reaction is zero order at still lower levels. The rate of sulfate reduction at constant initial sulfate and carbon substrate normalized to a constant bacterial density varies by a factor of 5 between 5° and 25°C (Kaplan and Rittenberg, 1964). Extreme pressure decreases the rate of sulfate reduction (Jannasch et al., 1971). Measurements of rates of sulfate reduction have utilized the change in concentration with time in laboratory-maintained mud samples (Nakai and Jensen, 1964; Martens and Berner, 1974; Goldhaber and Kaplan, 1975), or by analysis of pore water profiles using mathematical models (Ivanov, 1964). In rich sediments, a month may be required for extensive sulfate removal. However, the use of $^{35}SO_4=$ uptake has allowed detection of measurable rates much more rapidly in Black Sea sediments (Sorokin, 1962) and in oil well water (Ivanov, 1964). Rates in nature from 5.9×10^{-4} to 4×10^{-2} moles/liter/year have been recorded in the sea (Goldhaber and Kaplan, 1975).

These *Desulphovibrio* organisms are concentrated in the sediments (Kata et al., 1964), not the water column. Consequently our measurements involve the sediments. From measurements of total ATP in the sediments (ATP is a measure of viable bacterial mass), the richest area is in the uppermost centimeter, which contains 5 to 10 times the microbial mass of deeper layers (Christian et al., 1975). It was previously thought that the *Desulphovibrio* organsms required strict anaerobiosis for viability, but studies by Sand et al. (1975) have shown that exposure to air for as long as two hours will not decrease their viability.

The reduction of sulfate is stoichiometrically coupled with the generation of H_2S (Peck, 1974). The H_2S generally reacts with heavy metals to form insoluble sulfides, which give the black color to the muds in the bottom of ponds and lakes. In many studies only a small proportion of sulfate reduced can be recovered as free H_2S. The greater the rates of sulfate reduction, the greater the amount of iron pyruvate (FeS) recoverable in the sediments (Goldhaber and Kaplan, 1975). The free H_2S detected depends on the balance between rates of sulfate reduction and rates of sulfide complexing with heavy metals on one hand, and the oxidation of free sulfide aerobically by the Thiobacilli or

anaerobically in the light by the photosynthetic Thiorhodaceae on the other.

METHODS

Collection

Bottom samples were collected by Frank T. Manheim and immediately placed in equal volume of 6*N* HCl placed in glass bottles. The bottles were then placed in hermetically sealed cans and maintained at 4°C until hydrolysis.

Hydrolysis

Samples were removed from the containers and lyophilized for 24 hours at room temperature. Samples were then placed in test tubes with 6*N* HCl 20:1 (v/w) and sealed with a Teflon-lined screw cap. Sample sizes (dry weight) were 1-5 g for sediments, 0.5-1.0 g for plant litter, and 0.05 g for bacterial cultures. After hydrolysis at 105°C for 4.5 hours, the samples were cooled, filtered through coarse sintered glass filters into 250-ml round bottom flasks. Three washings of the hydrolysis vessel and filter were added to the flask. The samples were taken to dryness at 55°C with reduced pressure. Samples were then stored at -20°C in the flasks until further processing could occur.

Preparative Thick Layer Chromatography

Thick-layer 20 × 20 cm glass plates were coated with 1-mm-thick microcrystalline cellulose (Applied Science Laboratories, State College, Pa.) using a glass roller, a specially constructed trough, and a 20% aqueous suspension. Each sample was applied to a plate as a streak, 2.5 cm from the bottom, with a solvent of acetone 0.1*N* HCl, 9:1 (V/V). The successive applications must be thoroughly dried which often required use of the vacuum oven. The plates were developed by ascending chromatography through four successive cycles with a solvent of acetone, glacial acetic acid, water 9:1:1 (V/V). With each treatment the solvent front was allowed to migrate to the top of the plate, the plate dried, and the chromatography repeated.

Preliminary chromatography with standard mixtures showed that diamino pimelic acid (DAP) could be recovered from the R_f 0.1-0.2 band. This amino acid is characteristic of the wall protein of some bacteria. Dipicolinic acid (DPA) can be recovered from the solvent front. DPA is found only in the bacterial spore wall. Lactic acid, which would interfere with the colorimetric assay described below, runs with the solvent front. Muramic acid is found at R_f 0.5-0.57.

Recovery of the Muramic Acid

A band from R_f 0.35-0.70 was removed using a sealing tube with a coarse filter (Corning 59580) and vacuum (White and Frerman, 1967) and eluted with methanol:water 7:3 (V/V). The elution volume was concentrated in 20 mm diameter test tubes under a stream of nitrogen.

Colorimetric Assay

A modification of the colorimetric assay for lactic acid by Hadzja (1974) was utilized to take advantage of the alkaline lability of the ether bound in the muramic acid (Tipper, 1968).

Aliquots of 0.1, 0.4, and 1.0 ml were placed in screw cap test tubes, the volume made to 1 ml and 0.5 ml 1*N* NaOH added. After incubation for 30 min at 38°C, 10 ml of concentrated H_2SO_4 was added, the test tube tightly sealed with a Teflon-lined screw cap, and placed in a steam bath for 5 min. After cooling, 0.1 ml 4% W/V $CuSO_4$ and 0.2 ml 1.5% W/V *p*-hydroxydiphenyl in 95% V/V ethanol were added rapidly, the tubes tightly stoppered and shaken. After 30 min at 30°C the absorbance at 560 nm was measured, compared with standards of muramic acid (Sigma Chemical Co., St. Louis) or lithium lactate.

RESULTS

Authenticity of the Assay

The fraction recovered from the middle of thick layer plates was taken to dryness and re-suspended in a solvent which provided effective elution of muramic acid from a hydrolysis of bacteria or environmental samples from the Aminex A5 (particle size 13 ±2 μm) amino acid exchange resin (BioRad Corp., Richmond, California). The column was 53 × 0.9 cm, and was eluted at 42°C at a flow rate of 0.55 ml/min with a solvent made with LiOH • H_2O 12.74g, citric acid 9.09 g, thiodyglycol 2.5 ml., 10% BRIJ-35, 3 ml/liter made to *p*H 4.12. The putative muramic acid co-chromatographed with authentic muramic acid and disappeared when incubated at *p*H 7.4 at 30°C as does authentic muramic acid.

With this assay method hydrolysates of environmental and bacterial samples with 6*N* HCl at 105°C for periods between 0.75 and 8 hours were analyzed to maximize the recovery. Using a time of 4.5 hours with these conditions gave a recovery of 99 ±4.2% of authentic samples.

Interfering Substances and Reproducibility

Interfering substances were searched for in 10 samples each of marine sediments, estuarine plant litter samples, and cultures of *Escherichia coli*. They were hydrolyzed, then half the samples were incubated in 15% V/V ammonium hydroxide for 24 hours at 22°C. This destroyed any muramic acid present (Tipper, 1968). All samples were then fractionated by thick-layer chromatography, and the lactic acid derived from the muramic acid in the hydrolysate ran with the solvent front. The middle band was recovered and assayed colorimetrically. There was no lactate detected in the hydrolysates treated with ammonium hydroxide and then fractionated chromatographically. The values of muramic acid in the samples are given in Table 1. The reproducibility of the assay is also given in Table 1.

Muramic Acid Levels in Black Sea Sediments

In Table 2 values for the muramic levels of Black Sea sediments are given. The sediment was a metallic greenish color when removed from the glass bottles. The hydrolysate contained a large amount of yellow pigment and was remarkably hydroscopic. Colloidal material with the properties of elemental sulfur was present in the hydrolysate.

TABLE 1
Muramic Acid Content of Samples From Apalachicola Bay

Sample	Muramic Acid μg per g dry wt X ±σ
Oak litter	696 ± 96
Pine litter	611 ± 45
Sediment	34 ± 13
E. coli[a]	2630 ±266

[a]*E. coli* represents a laboratory culture.

TABLE 2
Muramic Acid Levels in Black Sea Sediments

Sample (Interval in cm)	Muramic Acid Content in μg/g dry wt
379A-19-5, 140-150	14.9
379A-38-7, 010-020	2.4
379A-45-2, 140-150	11.2
379A-64-4, 128-138	2.2
379B-03-1, 145-150	–
379B-04-1, 144-150	–
379B-04-4, 144-150	1.5
379B-06-4, 000-010	0.3
380 (Pressure Core Barrel)	1.8
380-10-3, 140-150	2.7

DISCUSSION

Muramic acid data indicate bacterial densities one-tenth to one-half that found in semitropical estuarine sediments. Assuming the organisms are like the *Desulphovibrio* organisms isolated from other habitats with a muramic acid content typical of gram negative organisms of about 3 μg muramic acid per g dry weight (Millar and Cassida, 1970), the sediments contain between 0.05% and 0.5% bacteria by weight. The environment of the Black Sea sediments may restrict the diversity and mass of the microflora. It is clear from the data that the microbial mass is not uniformly distributed.

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