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Improved Method Using Muramic Acid to Estimate Biomass of Bacteria in Sediments

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Summary. A method, which depends on the measurement of muramic acid content to estimate bacterial biomass, has been improved in sensitivity by two orders of magnitude. It is now applicable to any aquatic sediment, whereas previously it was mainly useful in the analysis of gut contents of deposit-feeding animals. Reduced NAD, a product of the oxidation of D-lactate derived from muramic acid, is assayed using bacterial luciferase. The amount of muramic acid in a number of terrestrial and marine bacteria was measured, and found to be lower than that obtained with the previous, less specific, assay procedure. The muramic acid content of a blue-green alga has been measured, thus allowing blue-green algae to be taken into account when estimating bacterial biomass. Experimental evidence is presented which shows that muramic acid in cell wall fragments of bacteria is rapidly degraded by microorganisms in a marine sediment.

Introduction

A correlation between muramic acid and carbon content of bacterial cells has been used to estimate biomass of bacteria (Moriarty, 1975). The method was developed to measure the importance of bacteria in the food of detritus-feeding animals, but was not very sensitive when applied directly to sediments. Another assay for D-lactate, which is more than 2 orders of magnitude more sensitive, is described below. The new assay has been tested with different types of bacteria (Gram negative and Gram positive) of marine and terrestrial origin and a blue-green alga. It was found that the content of muramic acid in the bacteria was less than that reported previously (Moriarty, 1975). Possible reasons for this discrepancy are discussed below. A new formula for estimating the amount of bacterial carbon in surface marine sediments is given in this paper.

One of the main assumptions made in applying this method, was that muramic acid in cell wall fragments of dead bacteria would not persist for long in sediments. To test this, cells walls of *Bacillus subtilis*, labelled with ^{14}C, were added to a marine sediment and the disappearance of ^{14}C-muramic acid was measured.

Materials and Methods

Microorganisms. The terrestrial bacteria were obtained from the Culture Collection, Department of Microbiology, University of Queensland. The marine bacteria were isolated from the top 1 cm of sediment on a sea-grass flat in Moreton Bay, Queensland. All bacteria were cultured as described previously (Moriarty, 1975). The blue-green alga, *Oscillatoria tenuis*, was obtained as an axenic culture from the Culture Collection of Algae, Indiana University, and grown on a solid medium (Allen, 1968). Filaments were carefully removed and freeze-dried.

Growth Phase Experiment. Two of the marine isolates (Nos. 6 and 11 in Table 1) were grown in 1 l of medium, aerated by bubbling, at 30°. Growth was monitored by optical density increase at 600 nm. Samples were withdrawn during the exponential phase and about 2 h after the start of the stationary phase of growth. Adenosine triphosphate was measured on 1 ml samples collected on a 0.45 µm filter as described by Hamilton and Holm-Hansen (1967). For muramic acid and carbon determinations, 200 ml samples were centrifuged at $10{,}000 \times g$ for 5 min, washed once in 100 ml of filtered sea-water and freeze-dried.

Carbon. Carbon was measured as described previously (Moriarty, 1975).

Muramic Acid. Hydrolysis was carried out as described previously (Moriarty, 1975).

D-*Lactate Assay.* The procedure is an adaptation of that described by Noll (1974) for L-lactate, in which the pyruvate is trapped as alanine by glutamate-pyruvate transaminase. The reduced NAD[1] is assayed with bacterial luciferase (Stanley, 1971). These references should be consulted for full details on the preparation and stability of reagents and other procedural aspects, although a few modifications have been made. It has not been possible to couple the two reactions in one vessel.

A small volume (up to 100 µl) of sample solution was placed in a small test tube. Distilled water was added to bring the volume up to 100 µl where necessary. Each test tube contained about 10 ng to 200 ng of D-lactate. A set of standards covering this range was also prepared, using lithium D-lactate. Immediately before use, a volume of the following reagents, sufficient for the number of samples desired, was prepared.

Glutamate buffer: 0.3 M, pH 9.0	0.5 ml/assay
NAD: 33 mg/ml in H_2O	10 µl/assay
D-LDH: 5 mg/ml	1 µl/assay
GPT: 10 mg/ml (80 units/mg)	0.5 µl/assay.

From a dispensing bottle 0.5 ml were mixed rapidly into each sample tube. The tubes were placed in a water bath at 30° for 15 min and then transferred to crushed ice. A volume of the following reagents, sufficient for triplicate analyses of each of the above samples, was prepared and equilibrated at 29°.

Phosphate buffer: 0.1 M, pH 7.5	2 ml/assay
2-mercaptoethanol	14 µl/assay
FMN: 5 mg/10 ml H_2O	10 µl/assay
Dodecyl aldehyde: saturated in ethanol	10 µl/assay.

Dodecyl aldehyde was suspended in ethanol and centrifuged immediately before use. Bacterial luciferase was prepared in phosphate buffer (1.5 mg/ml) with an equivalent amount of bovine serum albumen and kept on ice. Any material not dissolved after 15 min was removed by centrifuging at $3000 \times g$ for 5 min at 0°. The sensitivity of the assay depends on the amount of luciferase used; thus if samples contained about 10 to 50 ng lactate, 100 µl of this enzyme solution were used. For samples

[1] *Abbreviations:* ATP = adenosine triphosphate; FMN = flavin mononucleotide; GPT = glutamate pyruvate transaminase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2); D-LDH = D(−)-lactic acid dehydrogenase (D-lactate: NAD oxidoeductase, EC 1.1.1.28); MA = muramic acid; NAD = nicotinamide adenine dinucleotide (oxidised form); NADH = nicotinamide adenine dinucleotide (reduced form)

with 50 to 200 ng lactate, 50 µl were sufficient. Mercaptoethanol and bovine serum albumen stabilise the bacterial luciferase (Hastings and Gibson, 1963).

Two ml of the phosphate mixture were dispensed into a small polypropylene vial and luciferase was added and mixed. Then noting the time, 100 µl of the lactate reaction mixture were added. The tube was capped, mixed and placed in a scintillation vial. The counting sequence was started precisely 10 s (or a similar, reproducible interval) later. The scintillation counter (Packard Model 2250) was set up as follows: time 0.2 min; gain 100%; window 110–150; coincidence switch off.

Decomposition of Cell Walls. Bacillus subtilis cell walls, labelled with ^{14}C, were prepared as described elsewhere (Moriarty, 1976). A slurry of surface sediment was obtained from a mangrove area on Moreton Bay, Queensland. About 2 mg dry weight with an activity of about 1×10^6 cpm, were vortex mixed with 10 ml of mud for 30 s. Three ml were removed immediately and frozen. A piece of filter paper, moistened with 20% w/v KOH, was placed in a cup inside the flask of sediment. The flask was shaken at 30°. The filter paper was replaced at intervals, in order to monitor $^{14}CO_2$ release. After 24 h and 48 h, further 3 ml aliquots were removed and frozen. These samples were freeze-dried and then hydrolysed to form D-lactate from any muramic acid present. Each of the hydrolysates was subdivided. In one half of each, the D-lactate was converted to pyruvate, using D-LDH and 3-acetylpyridine NAD (Maurer and Poppendiek, 1974). D-LDH was then denatured by the addition of 1 M HCl to pH 2. Sodium citrate (0.5 M) was added, raising the pH to 6.0, followed by 1 mg/5 ml of pyruvate decarboxylase (2-oxo-acid carboxylyase, EC 4.1.1.1). Filter paper, moistened with KOH, was placed in a cup in the flask in order to collect $^{14}CO_2$ released from pyruvate. The flask was then sealed and shaken at 30° for 1 h. The reaction was terminated by injecting 2 M H_2SO_4 into the flask, and after shaking for a further 1 h, the paper was removed. Filter papers were counted by liquid scintillation, with Triton X100 in the scintillant fluid (Turner, 1971). As a control to allow for $^{14}CO_2$ from sources other than ^{14}C-muramic acid, the other half of each hydrolysate was treated as above, but D-LDH was omitted.

Bovine serum albumen, bacterial luciferase and pyruvate decarboxylase were obtained from Sigma Chemical Co., St. Louis, USA; D-LDH and GPT from Boehringer, Mannheim, F.R.G.; and dodecylaldehyde from Koch Light Labs., Colnbrook, U.K.

Results

The Gram negative bacteria, both marine and terrestrial, differ by about two fold in the ratio of muramic acid to mgC (Table 1). Much of this difference is due to variation in the sizes of the cells. The marine organisms and *Pseudomonas fluorescens* and *Proteus vulgaris* all have about the same amount of muramic acid per unit surface area. *Escherichia coli, Enterobacter aerogenes* and *Serratia marcescens* have about twice as much, which suggests that their murein layer is twice as thick. These results should be interpreted with caution, however, because it was difficult to measure the cell diameters accurately and it was assumed that the cells were perfect cylinders. The Gram positive bacteria vary considerably in their content of muramic acid. Of the marine bacteria, organism No. 11 (a *Bacillus*) stained strongly Gram positive and has a high content of muramic acid, whereas organisms 9 and 10 were Gram negative except in the early stages of growth when they were Gram positive. Isolate No. 9 exhibited a change in morphology during the cell cycle, with rods present during the exponential phase and cocci during the stationary phase.

The ratio of muramic acid to mgC in the blue-green alga, *Oscillatoria tenuis*, is similar to that of the Gram negative bacteria. Its murein layer is much thicker, however, as it has about 10 times as much muramic acid per unit area (Table 1). The amount of muramic acid per cell was measured by counting the number

Table 1. Muramic acid content of some terrestrial and marine bacteria. Range of variation: ±10% for 3 determinations each. Bacteria were harvested during the exponential phase of growth, except the coccoid form of No. 9 which was obtained during stationary phase. The ratios of muramic acid to surface area are an approximate calculation from the values of MA/mgC, using cell diameters measured in a phase contrast microscope, and assuming specific gravity is 1.1; C is 45% of dry weight, and dry weight is 20% of wet weight

Terrestrial bacteria	μgMA/ mgC	fgMA/ μm²	Marine bacteria	μgMA/ mgC	fgMA/ μm²
Gram negative			Gram negative		
Escherichia coli	10	0.30	Isolate No. 1	10	0.15
Enterobacter aerogenes	10	0.30	Isolate No. 2	9	0.13
Serratia marcescens	10	0.27	Isolate No. 3	9	0.15
Proteus vulgaris	10	0.15	Isolate No. 4	7	0.15
Pseudomonas fluorescens	7.5	0.15	Isolate No. 5	7	0.13
			Isolate No. 6	6	0.13
			Isolate No. 7	5	0.13
			Isolate No. 8	5	0.13
Gram positive, pleomorphic			Weak Gram positive or variable		
Arthrobacter globiformis	27	0.42	Isolate No. 9 (rod form)	13	0.20
			Isolate No. 9 (cocci)	16	0.20
			Isolate No. 10	14	0.35
Gram positive			Gram positive		
Bacillus subtilis	40	0.90	Isolate No. 11 (*Bacillus* sp.)	44	1.10
Micrococcus aurantiacus	57	1.00			
Blue-green algae					
Oscillatoria tenuis	11	1.60			

Table 2. Comparison of biomass and numbers with muramic acid and ATP content of two marine bacteria during growth. Range of variation in C, MA and ATP: ±5%; pseudomonad viable counts: ±20%; total counts are given for the *Bacillus*, but these are approximate because the cells occurred in pairs or short chains, and clumped together

Growth phase	*Bacillus* sp.		Pseudomonad	
	Exponential	Stationary	Exponential	Stationary
Number/ml	3×10^{8}	1×10^{9}	8×10^{8}	2×10^{9}
Carbon mg/cell	3×10^{-10}	3×10^{-10}	1×10^{-10}	1×10^{-10}
Muramic acid				
μg/mgC	44	39	6.5	4.7
μg/cell	1.4×10^{-8}	1.1×10^{-8}	6×10^{-10}	5×10^{-10}
ATP				
μg/mgC	3	2.8	5	5
μg/cell	3×10^{-9}	3×10^{-9}	0.5×10^{-9}	0.5×10^{-9}

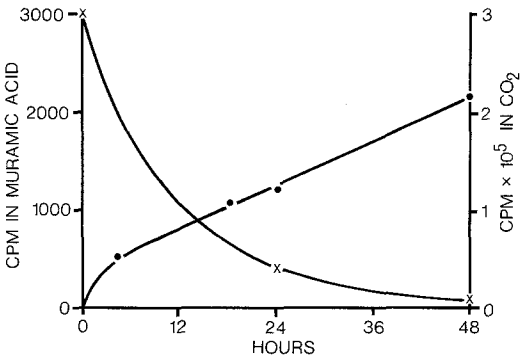

Fig. 1. Decomposition of ^{14}C labelled *Bacillus subtilis* cell walls in a marine sediment, monitored by the release of $^{14}CO_2$ (●) and the decrease in ^{14}C-muramic acid (×). ^{14}C-muramic acid was measured by enzymic release of $^{14}CO_2$ from the carboxyl group on its D-lactate moeity (see "Materials and Methods")

of cells in a portion of a sample that was subsequently assayed for muramic acid. A value of 1×10^{-7} µg MA cell was obtained (standard error: $\pm 0.2 \times 10^{-7}$, 10 counts).

The relationship between muramic acid and ATP content with biomass of two different marine bacteria was measured at two stages in the growth cycle (Table 2). It can be seen that the muramic acid content falls after the exponential phase, but the small change would have little effect on biomass estimation. The values calculated from the number of cells present, are given for comparison with enumeration methods for estimating biomass, but because it was difficult to obtain accurate numbers, these values are only approximate.

The cell walls of *Bacillus subtilis*, when mixed with a sediment, are metabolised by the microorganisms present (Fig. 1). In particular, about 90% of the muramic acid in the labelled wall material was degraded within 24 h. The $^{14}CO_2$ from pyruvate in the controls was about 10% of that from muramic acid at zero time.

Discussion

Assay Procedure. The values for the ratio of muramic acid to carbon (Table 1) are in general about half of those reported previously for the same bacteria (Moriarty, 1975). The method described above for the assay of D-lactate is much more sensitive than that used previously. It is also specific for NADH, whereas previously any reaction leading to an absorbance change at 340 nm would interfere. The discrepancy probably is due to other reactions in the complex bacterial hydrolysate which cause a change at 340 nm. Such changes could be caused by impurities in the D-LDH preparation. The values reported above have been checked by the use of internal standards (NADH and D-lactate). They agree reasonably well with values for muramic acid per unit dry weight of cells given by Millar and Casida (1970).

Precision in pipetting and reaction timing are essential for reproducible results with bacterial luciferase. To avoid quenching of light output and inhibition of this enzyme, the amount of hydrolysed bacteria in the LDH-GPT reaction mixture should not exceed about 100 µg dry weight per 0.5 ml. This can be checked by using internal standards or two different volumes of bacterial hydrolysate.

Two different volumes (say 20 µl and 100 µl) of each hydrolysed sediment sample should also be assayed, to check whether the various enzymes are being inhibited. If inhibition is occurring, the samples should be diluted.

The ultimate sensitivity is limited to about 10 ng lactate per assay. It could be improved by purifying further the commercial enzyme preparations and by using a scintillation spectrometer which can integrate all or most of the light flash from the luciferase. At low levels of NADH, the light flash decays within a few seconds. Much larger amounts of lactate can be measured by the technique described here. I have measured from 200 to 1 µg of D-lactate per assay by using twice the concentration of D-LDH and GPT, incubating for 20 min at 30° and by using 20 µl of luciferase. With larger amounts of D-lactate and thus NADH, the light flash reaches a peak more slowly. It is necessary to check, therefore, that the time delay between adding the final solution and starting the counting sequence is longer than the time taken for the light flash to reach a peak in all samples. With this system, it is better to integrate a portion of the decay period of light emission, rather than the initial increase in light emission.

Biomass Estimation. As pointed out previously (Moriarty, 1975) only an approximate indication of the proportions of the main groups of bacteria present in a natural environment is needed to give a reasonable estimate of biomass. The formula given previously (Moriarty, 1975) must be modified according to the values in Table 1. In marine sediments, if most of the bacteria are Gram negative or weak Gram positive (with low muramic acid content), a combined value of about 12 µg MA/mgC for these organisms would be sufficiently accurate for estimating biomass. Thus the formula should be modified for this environment to:

$$C = 1000\ \mathrm{MA}/(12n + 40p)$$

where n is the proportion of Gram negative or weakly Gram positive bacteria and p is the proportion of strongly Gram positive bacteria.

For surface marine sediments, where strongly Gram positive organisms (with a high content of muramic acid) are probably not abundant, a value of 15, substituted for the denominator, would accommodate some variation in population composition and includes blue-green algae. Blue-green algae could be counted using a fluorescent microscope with epi-illumination, and a corresponding estimate of their muramic acid content deducted from the total before applying the above formula. For example, if coccoid cells 6 µm in diameter are present, and it is assumed their murein layer is about the same thickness as *Oscillatoria tenuis*, it can be calculated that each cell would contain about 2×10^{-7} µg muramic acid. There is a possibility that the electron microscope could be used to obtain a better indication of the proportion of Gram negative bacteria in a sediment population. Weibull (1975) examined thin sections of bacteria concentrated from lake water and found that the majority were Gram negative.

Bacterial biomass has been measured in the food of two deposit-feeding animals (Moriarty, 1976). Although the method used to measure muramic acid overestimated the amount present, the ratio of bacterial carbon to total organic carbon is still about the same.

Muramic Acid Decomposition. In using muramic acid to estimate biomass of bacteria in sediments, I assumed that muramic acid in cell wall fragments from dead bacteria did not persist for long (Moriarty, 1975). The experiment described above (Fig. 1) shows that microorganisms in the sediment rapidly degrade muramic acid. The actual amount of wall material added to the sediment was large in comparison to the size of the living population, and yet most of the muramic acid was utilised within 24 h. Autolytic enzymes, inactivated in the above experiment, would be expected to increase the rate of cell wall decomposition. Harrison and Mann (1975) in an experimental study on sea-grass decomposition, have shown that protozoa graze heavily on the bacterial population. As argued earlier (Moriarty, 1975), this should reduce the proportion of senescent bacteria and thus cell wall fragments. Thus it is unlikely that muramic acid in cell wall fragments is a large source of error in the estimation of biomass.

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