APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1978, p. 251-257
0099-2240/78/0035-0251$02.00/0

Vol. 35, No. 2
Printed in U.S.A.

Recovery of Poly-β-Hydroxybutyrate from Estuarine Microflora

JEAN S. HERRON, JOHN D. KING, AND DAVID C. WHITE*
Department of Biological Science, Florida State University, Tallahassee, Florida 32306

Received for publication 12 September 1977

Poly-β-hydroxybutyrate (PHB) is a uniquely procaryotic endogenous storage polymer whose metabolism has been shown to reflect environmental perturbations in laboratory monocultures. When hydrolyzed for 45 min in 5% sodium hypochlorite, PHB can be isolated from estuarine detrital microflora in high yield and purified free from non-PHB microbial components. Lyophilization of frozen estuarine samples shortens the exposure time to NaOCl necessary for maximal recovery. Lyophilized samples of hardwood leaves, *Vallisneria*, and the aerobic upper millimeter of estuarine muds yielded PHB. The efficiency of incorporation of sodium [1-^{14}C]acetate into PHB is very high and is stimulated by aeration. PHB was not recovered from the anaerobic portions of sediments unless they were aerated for a short time. Levels of PHB in the detrital microbial community do not correlate with the microbial biomass as measured by the extractible lipid phosphate, suggesting that PHB-like eucaryotic endogenous storage materials may more accurately reflect the metabolic status of the population than its biomass.

Poly-β-hydroxybutyrate (PHB) is an endogenous reserve polymer found widely in both gram-positive and gram-negative procaryotes (10, 11). This polymer has been detected in numerous heterotrophic and autotrophic aerobic eubacteria, photosynthetic anaerobic bacteria (11), gliding bacteria (28), actinomycetes (9, 17), and cyanobacteria (6). Except for the E strain of *Clostridium botulinum* and *Rhodospirillum rubrum* grown heterotrophically in the dark, PHB has not been detected in obligate anaerobes (14, 39). The presence of high levels of PHB in cells increases survival during nutrient limitation in *Micrococcus halodenitrificans* (33), *Hydrogenomonas* (29), *Sphaerotilus discophorius* (38), *Pseudomonas* (47), and *Azotobacter* (35), where PHB is used both as a carbon and energy source. It serves as an endogenous energy source for *Bacillus megaterium* (11) and *Pseudomonas saccharophilia* (13). In *Rhodospirillum rubrum*, PHB serves as a carbon source in the dark (36) and an energy source in the dark when activated with air (4).

Floc formation by *Zoogloea* (7, 8) and *Pseudomonas* (47) has been linked closely with PHB synthesis but has been shown to be an absolute requirement in *Zoogloea* (12).

During the special conditions of nitrogen fixation by aerobic nitrogen fixers, synthesis of PHB serves as a terminal electron acceptor that is of great survival value. It promotes electron transport at the low oxygen tensions necessary to protect the nitrogen reductase system (16).

One of the interesting functions of PHB is its relationship to the process of spore or cyst formation. It accumulates before sporulation in the bacilli (20, 34) and serves as an energy and carbon source for the terminal sporulation reaction (26). Accumulation just before use in sporulation occurs in *C. botulinum* E (14) and in the formation of cysts by *Azotobacter* (37). PHB accumulation precedes its utilization and antibiotic release in *Streptomyces antibioticus* (21) and *Actinomycetes* (17).

In the precisely controlled environment of the chemostat, with an adequate energy source, limitation of oxygen or nitrogen in *Hydrogenomonas* (29, 30), oxygen and nitrogen in *Azotobacter beijerinckii* (16, 31), and nitrogen, sulfur, potassium, or carbon in *B. megaterium* (45) can cause PHB accumulation.

The sensitivity of the level and metabolism of PHB to environmental perturbation in laboratory monocultures prompted an examination of the estuarine detrital microflora to determine whether this polymer was present and, if so, whether its level paralleled the microbial biomass or could be a more sensitive measure of the physiological status of the population.

MATERIALS AND METHODS

Reagents. Analytical reagent-grade solvents (obtained from Mallinckrodt or Fisher Scientific Co.) were used without further purification. Sodium

[1-^{14}C]acetate (New England Nuclear Corp., Boston, Mass.) was used. Other reagents have been discussed previously (19, 32, 41).

Preparation of estuarine detritus. Oak leaves (*Quercus virginiana*), *Vallisneria americana* leaves, sweet gum leaves (*Liquidamber styraciflua*), pine needles (*Pinus elliottii*), polethylene sheets cut in the shape of oak leaves, and extruded polyvinyl chloride needles were incubated in baskets in Apalachicola Bay, Fla., in the summer and fall of 1977 (3, 25, 43). Leaf samples were cut into disks 6.5 mm in diameter, and needles were cut into 1.5-cm lengths after return to the laboratory.

Preparation of ^{14}C-labeled materials. *Escherichia coli* B was incubated in 250 ml of nutrient broth containing 50 μCi of [*U*-^{14}C]glucose for 18 h at 37°C. The cells were recovered by centrifugation and washed with 50 mM phosphate buffer (pH 7.6). The activity was 7.94×10^5 dpm/100 mg (wet weight). *B. megaterium* subsp. *globigii* was grown in the nitrogen-free medium of Macrae and Wilkinson (24) with the substitution of 0.01% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) for the Casamino Acids. Sodium acetate (100 mM) and glucose (5 mM) were used as carbon sources in 1,400 ml of medium in 2.5-liter, low-form culture flasks incubated with agitation at 37°C. A 1% (vol/vol) inoculum of an 18-h liquid culture was used. The presence of PHB was monitored by the appearance of sudanophilic granules (5) and isolated from the bacteria by the method of Williamson and Wilkinson (46).

Radioactivity was determined by scintillation spectrometry as described previously (18, 19, 25).

[^{14}C]PHB was prepared as described above with 1.3 mCi of sodium [1-^{14}C]acetate in a liter of medium (10 mM sodium acetate plus 50 mM glucose). The purified [^{14}C]PHB had a specific activity of 5,000 dpm/μg.

PHB assay. Between 25 and 50 leaf disks or fragments (8 to 15 mg [dry weight]) were placed in a 50-ml round-bottomed centrifuge tube with 5 ml of 5% NaOCl (Mallinckrodt AR) for 30 to 60 min at 37°C in a rotary shaker at 100 to 200 rpm. The polymer was then extracted with two 5-ml portions of chloroform, with thorough mixing and centrifugation when necessary. The chloroform was transferred into a screw-cap test tube (10 by 25 mm) marked at 5 and 10 ml. No leaf detritus was transferred, as it could have obscured the spectrophotometric assay. The solvent was removed by evaporation, 3 ml of water was added, and the tube was mixed vigorously. Then 3 ml chloroform was added, and the mixture was shaken with a Vortex mixer and centrifuged. The upper water layer was removed. Any material at the interface was not removed. A 3-ml water wash was repeated and removed, and the chloroform was evaporated away. Two to 3 ml of 95% ethanol was added, mixed thoroughly, centrifuged, and removed; care was taken not to touch the sides or bottom of the tube with the pipette. The 95% ethanol wash was repeated, followed by two washes with diethyl ether. The purified polymer remaining in the tube was dissolved in warm chloroform for spectrophotometric assay as described by Law and Slepecky (22). Portions of up to 0.1 ml were placed on paper disks (2 by 2 cm), and the ^{14}C was determined. If only radioactivity was to be determined, the original chloroform extract was added to filter paper disks (2 by 2 cm; Whatman 3MM) suspended on a pin board. The PHB was then bonded to the paper by pouring 0.1 ml of warm chloroform over the surface and heating for 5 to 10 min at 80°C (40). The paper disks were then washed with water, alcohol, and ether in petri dishes before the ^{14}C was determined.

RESULTS

Recovery of added [^{14}C]PHB. Preliminary determinations of recovery from the detrital microflora involved the addition of 10 μg of [^{14}C]PHB (specific activity, 5,000 dpm/μg) prepared from *B. megaterium* to oak leaf disks that had been incubated in Apalachicola Bay for 28 days. The extract was collected in chloroform and transferred to a centrifuge tube. After the chloroform was removed in a stream of nitrogen, the PHB was washed with diethyl ether, redissolved in chloroform, and added to a paper disk, and the radioactivity was determined. From these experiments, extraction with 5% NaOCl for 1 h at 37°C gave a recovery of 96%. Longer incubations with NaOCl at 37°C or 1, 2, or 6 N HCl at 100°C for 2 h yielded lower recoveries.

Effect of lyophilization on extraction efficiency. Samples of oak leaves were incubated in the estuary for 24 days and then held in the laboratory with vigorous aeration for 28 days. The aeration in the laboratory greatly increased the PHB levels. Five batches of 50 disks chosen randomly from a pool of 1,000 were frozen, lyophilized, and compared with five batches that were extracted wet. The absorbance at 235 nm for the lyophilized disks was 0.792 ± 0.103, compared with 0.802 ± 0.046 for the disks extracted wet. This gave values of 246.1 μg of PHB/g (dry weight) for lyophilized disks and 249.0 μg of PHB/g (dry weight) for the wet disks. The absorption spectrum at wavelengths shorter than 235 nm was much less with the lyophilized preparation, indicating a cleaner preparation from the lyophilized material.

Extraction efficiency of endogenously generated detrital [^{14}C]PHB. Oak leaf detritus was incubated with sodium [^{14}C]acetate, recovered, frozen, and lyophilized; the PHB was isolated by several methods. The most effective extractant was 5% NaOCl. The Bligh and Dyer extraction (2) modified for maximum extraction of lipids from the detrital microflora (19) released 42.3% of the [^{14}C]PHB recovered with NaOCl. Extraction by chloroform at 37°C for 60 min released 42% of the [^{14}C]PHB.

The incubation time necessary for maximal release of endogenously generated [^{14}C]PHB by 5% NaOCl was between 20 and 50 min (Fig. 1).

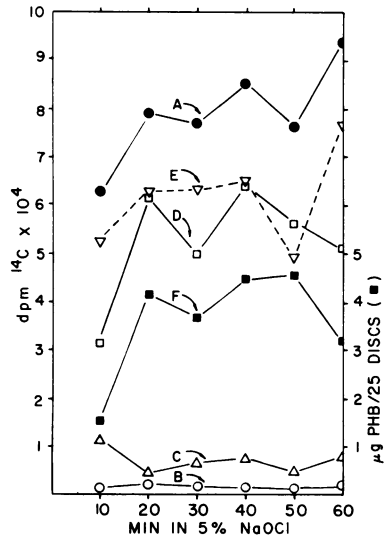

FIG. 1. *Release of endogenously generated [^{14}C]PHB from lyophilized detrital microflora by incubation in 5% (wt/vol) sodium hypochlorite at 37°C. Curve A: ●, ^{14}C in chloroform extract; curve B: ○, ^{14}C removed from chloroform extract with water; curve C: △, additional ^{14}C removed by extraction with diethyl ether; curve D: □, purified [^{14}C]PHB recovered in the second chloroform solution; curve E: ▽, purified [^{14}C]PHB carried on filter paper through the purification; curve F: ■, micrograms of purified PHB released per 25 oak leaf disks.*

The [^{14}C]PHB released into the NaOCl and extracted into chloroform is illustrated in Fig. 1. The ^{14}C recovered in water washes and ether washes indicated no significant degradation of the PHB polymer into more soluble fragments in the time interval measured. The amount of purified [^{14}C]PHB recovered by the standard method compared to the amount of [^{14}C]PHB fixed to filter paper squares before the washings and the total purified PHB measured spectrophotometrically.

Purification of PHB. The addition of *E. coli* grown with [^{14}C]glucose to the detrital microflora was used to test the efficiency of the purification procedure's removal of non-PHB microbial components. The bulk of the ^{14}C-labeled *E. coli* (about 77%) was not extracted into the chloroform (Table 1). The successive water washes removed 30.5, 6, and 2% of the ^{14}C-labeled *E. coli* from the chloroform-soluble material. The 95% ethanol washes removed 40 and 1.4% of the ^{14}C left in the water-washed chloroform extract. Neither the acetone nor diethyl ether removed ^{14}C-labeled *E. coli* components. Only 0.1% of the ^{14}C-labeled *E. coli* components contaminated the purified PHB from the detrital microflora. The ^{14}C recovered in the successive steps accounted for 102% of the ^{14}C-labeled *E. coli* added.

TABLE 1. *Purification of PHB from the detrital microflora in the presence of added ^{14}C-labeled E. coli or [^{14}C]PHB prepared from B. megaterium*

Purification step	Substance recovered (dpm × 10^3)		
	^{14}C-labeled *E. coli*	[^{14}C]PHB	[^{14}C]PHB
5% NaOCl[a]	1,598	132	132
$CHCl_3$[b]	364	126	124
Water[c]	111	7.6	
Water	21.6	0	
Water	7,900	0	5.4
95% Ethyl alcohol[d]	143	6.7	4.26
95% Ethyl alcohol	5.1	4.1	4.65
Acetone[e]	0	19.4	—[f]
Acetone	0	28.7	—
Ethyl ether[g]	0	4.38	2.28
Ethyl ether	0	0	3.1
Ethyl ether	0	2.4	—
$CHCl_3$[h]	1.56	39	100

[a] A total of 30 disks (9.6 mg [dry weight]) of lyophilized oak leaves that had been incubated in Apalachicola Bay for 20 days, to which was added *E. coli* grown with ^{14}C (left-hand column) or purified [^{14}C]PHB from *B. megaterium* (middle and right-hand columns) was used. (In the right-hand column the acetone extractions from the [^{14}C]PHB were omitted.) PHB was extracted from the disks with 4 ml of 5% (wt/vol) sodium hypochlorite at 37°C with shaking for 60 min. At each step, portions were removed and transferred to scintillation vials, the solvent was evaporated, and the radioactivity was determined.

[b] The hypochlorite extract was extracted with two 5-ml portions of chloroform.

[c] The $CHCl_3$ was removed in a stream of air, and the pellet was washed with 30 ml of water three times.

[d] Pellet was washed twice with 3.0 ml of 95% ethyl alcohol.

[e] Pellet was washed twice with 2.5 ml of acetone.

[f] —, Acetone and ether extractions omitted from [^{14}C]PHB.

[g] Pellet was washed three times with 2.0 ml of diethyl ether.

[h] Pellet was suspended in 1 ml of chloroform and transferred to a scintillation vial, chloroform was removed in a stream of nitrogen, scintillation fluid was added, and radioactivity was determined.

Losses of added [^{14}C]PHB during purification. The purification procedure with purified [^{14}C]PHB as a tracer is illustrated in Table 1. Ninety-six percent of the added [^{14}C]PHB was extracted by chloroform from the hypochlorite-releasing treatment. Only 30.8% of the chloroform-soluble [^{14}C]PHB was recovered after purification when acetone washes were included. Spectral analysis after hydrolysis and dehydration of the acetone washes showed the characteristic spectral maximum at 235 nm of hydrolized-dehydrated PHB. When water, ethyl alcohol, and diethyl ether were used and the acetone washes were omitted, 80.5% of the [^{14}C]PHB in the chloroform extract was recovered. This compares to the average recovery of 79% with endogenously generated [^{14}C]PHB (Fig. 1).

Absorbance spectrum. The absorbance of the purified PHB isolated from *B. megaterium*

measured in concentrated H_2SO_4 after heating for 10 min at 100°C is illustrated in Fig. 2. This absorbance spectrum was measured on 2.79 μg of PHB/ml. Ten replications gave an absorbance at the maximum (235 nm) of 0.459 ± 0.006.

At PHB concentrations of 0.2 to 1.0 μg/ml, there was a linear increase in absorbance (correlation coefficient, $r^2 = 0.999$), with $\epsilon 1\% = 1{,}650$, where 6.06 μg of PHB/ml equals an absorbance of 1.0.

Identification of PHB derived from detrital microflora. A sample of PHB purified from the oak leaf detritus had the solubility properties of the PHB isolated from *B. megaterium* (46). It was soluble only in chloroform and essentially insoluble in diethyl ether or water (Table 1), and formed the characteristic sticky white precipitate that was difficult to transfer quantitatively from glass containers. Putative microfloral PHB was dissolved in concentrated H_2SO_4 and heated at 100°C for 10 min. The resulting dehydrated sample was then split into two 2.5-ml portions. To one sample was added 2.5 ml of water; the other received 2.5 ml of H_2SO_2. The ultraviolet absorbance spectra (Fig. 2) indicate a hydrolysis and reversible dehydration identical with those of purified PHB.

Analysis of the dehydration-hydration difference spectra may be useful in samples difficult to purify from ultraviolet-absorbing contaminants. The change in absorbance at 235 nm between hydrolysis, dehydration, and rehydration is 0.702 times the absorbance with the standard procedure. The $\epsilon 1\%$ of the difference in absorbance at 235 nm for dehydration-hydration is 579.6 for a sample in concentrated H_2SO_4.

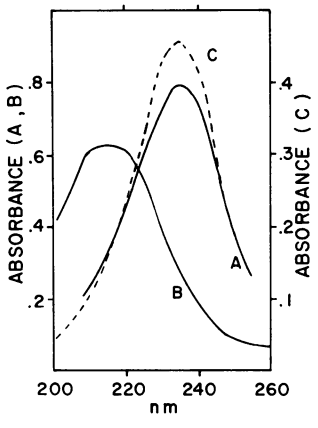

FIG. 2. *Absorption spectra of purified PHB in concentrated H_2SO_4 after heating for 10 min at 100°C. Curve A: Sample purified from oak leaf detrital microflora diluted by half into concentrated H_2SO_4 (dehydrated). Curve B: Oak leaf detrital PHB in concentrated H_2SO_4 diluted by half into water (hydrated). Curve C (read on right-hand axis): Purified PHB (2.79 μg/ml) from B. megaterium (dehydrated).*

Reproducibility. The variance of the spectrophotometric assay of PHB ranged between 6 and 14% of the mean. Determinations of ^{14}C among portions of the same sample purified by extractions with water, ethanol, and diethyl ether had a variance between about 5 and 8% of the mean.

Recovery of PHB from environmental samples. The applicability of this assay system to a number of components in the estuary was examined. Detrital hardwood leaf input enters this estuary with the river flow. In this study, lyophilized oak leaves isolated from the estuary in the summer yielded 15 to 30 μg of PHB/g (dry weight). When the leaves were returned to the laboratory and incubated with vigorous aeration for a few days, the PHB level increased 5- to 10-fold. Sweet gum leaves (*L. styraciflua*) also are a prominent detrital component and yielded to 50 to 70 μg of PHB/g (dry weight) from lyophilized samples. Benthic macrophytes, principally *Vallisneria americana* collected from the intertidal zone in August and lyophilized, yielded 140 μg of PHB/g (dry weight).

Mud samples were taken with a gravity corer at a depth of 1.5 m. The core plastic liners (6 cm in diameter) were removed and frozen. The frozen cores were then cut in 1-cm sections, with the surface section containing 1 to 2 mm of mud, and lyophilized. From the lyophilized sediments, 2- to 5-g samples of mud were treated with NaOCl and extracted with chloroform. The mixture was centrifuged and a sample was removed for purification. The uppermost millimeter of the surface yielded 0.14 μg of PHB/g (dry weight). When a surface mud sample was vigorously aerated for 2 days and the suspension was extracted, the mud microflora showed a 10-fold increase in PHB. No PHB (<0.001 μg/g [dry weight]) was detected in the anaerobic sections of the core.

Pine needles (*Pinus elliottii*), wet or lyophilized, yielded analytes not suitable for spectrophotometric assay, as an ultraviolet-absorbing pigment obscured the colorimetric assay for PHB despite many solvent washing combinations. The same was true for the detrital microflora of extruded polyvinyl chloride needles.

Synthesis of PHB by detrital microflora. The microflora of oak leaves formed after incubation in Apalachicola Bay for 7 weeks readily incorporated ^{14}C from sodium [1-^{14}C]acetate into PHB (Fig. 3). By maintaining the ^{14}C in filtered water within 10% of the initial activity with repeated additions of ^{14}C radioactive precursor, an exponential rate of incorporation was maintained for 5 h.

In a 3-h incorporation experiment in which 1.28×10^8 dpm sodium [1-^{14}C]acetate in 500 ml of bay water was incubated with 9.26 g (dry weight) of detritus, about 23% of the total ^{14}C added to the culture was recovered in the PHB, compared with 1.95% recovered in the phospholipid and 1.2% in the neutral lipid.

PHB and microbial biomass. Lipid phosphate is an accurate measure of microbial mass in monocultures (41, 44) that also correlates well with extractible adenosine 5'-triphosphate, total respiratory capacity, and muramic acid in the detrital microflora (42).

By using the extractible lipid phosphate from a series of estuarine detrital microbial samples to estimate the biomass, the relationship between microbial mass and PHB was determined. Previous work has shown that 2-week incubation of dried leaves in the estuary results in predominantly bacterial growth (25). A series of five biweekly samples of *Vallisneria* leaves and polyethylene sheets cut into the shape of leaves was incubated in the estuary. (A linear correlation coefficient [r] of +0.13, indicating essentially no correlation, was found between PHB and lipid phosphate recovered from *Vallisneria* leaves.) The *Vallisneria* leaves showed values of 3.39 ± 2.57 μg of PHB per μmol of lipid phosphate ($\bar{x}$ ± SD). The microflora associated with the polyethylene surface showed a negative linear correlation coefficient (r) of −0.40, with values of 14.3 ± 18.5 μg of PHB per μmol of lipid phosphate.

DISCUSSION

A polymer with the solubility characteristics of authentic PHB (Fig. 1, Table 1), which gives the characteristic derivative on hydrolysis and dehydration (Fig. 2) and into which sodium [1-^{14}C]acetate can efficiently be incorporated, can be quantitatively isolated from the detrital microflora and purified from the ^{14}C-labeled components of a non-PHB-containing bacterium (Table 1).

In monocultures of *Azotobacter*, lyophilization of the frozen sample results in the release of a longer polymer in a shorter period of time (27). In the detrital microflora, lyophilization results in an equivalent yield of PHB, with shorter incubation time in NaOCl and a much purer product (as judged by the absorption spectrum in concentrated sulfuric acid) than does the extraction of fresh material. The increase in the efficiency of extraction apparently results from there being less time for breakdown into shorter polymers of PHB (1, 23). The shorter the polymer, the greater the losses into acetone and 95% ethanol during the purification procedure. The fact that environmental samples can

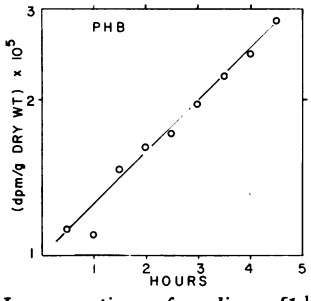

FIG. 3. *Incorporation of sodium [1-^{14}C]acetate into PHB by detrital microflora. Oak leaves incubated in Apalachicola Bay for 7 weeks were recovered, randomly cut into 5,000 6.5-mm disks, and incubated in 500 ml of bay water for 6 h in the presence of 100 μCi of sodium [1-^{14}C]acetate at 25°C with aeration. The radioactivity of filtered bay water was maintained within 10% of the initial value by periodic additions of more ^{14}C. Samples were removed, the PHB was extracted and plated on filter paper disks that were washed with water, 95% ethanol, and diethyl ether, and the ^{14}C was determined in the scintillation spectrometer. Note the logarithmic scale on the abscissa.*

be frozen in the field makes analysis considerably more convenient and probably more characteristic of the actual state of the organism, because fewer alterations occur during the return from the field to the laboratory.

Acetate and short-chain fatty acids are selectively incorporated into PHB in *Nocardia* (9), *Rhodospirillum rubrum* (36), *Chlorogloea fritschii* (6), and *Hydrogenomonas* (15). Up to 70% of added sodium [1-^{14}C]acetate is incorporated into PHB during its period of most rapid synthesis in *B. cereus* (26). Thus it is no surprise that sodium [1-^{14}C]acetate is 10 times more efficiently incorporated by the detrital microflora into PHB than into the lipid fatty acids. The facile incorporation into PHB by the detrital microflora makes studies of PHB metabolism in the environment relatively easy.

PHB can be recovered from several important detrital inputs into this subtropical estuary (Apalachicola Bay). It is also found in the aerobic zone above the redox potential discontinuity in the sediments. It is not detected in the anaerobic zones of the sediments, although aeration of these sediments results in its formation.

The uneven distribution of PHB in the estuarine environment and the fact that the synthesis of PHB in bacterial monocultures can be greatly stimulated under various nongrowth conditions in some of the procaryotic protists (11, 45) suggest that PHB would not be a consistent measure of microbial biomass. Comparison of estuarine detrital samples showed no consistent relationship between phospholipid con-

tent and PHB in samples recovered from the environment. PHB, then, is not a useful measure of microbial biomass in the complex, uncontrolled conditions of field studies. However, the sensitivity of PHB metabolism to specific perturbations, such as aeration with adequate carbon sources in monocultures (16, 30, 33), and the rapid increase in PHB levels detected with aeration in both detrital and sedimentary samples may prove PHB to be a sensitive measure of the physiological status of the community. Preliminary investigations suggest that perturbations in the environment (such as anoxia) that do not affect the microbial biomass in the short term markedly slow the metabolism of PHB. Further insights into the effects of various environmental perturbations on PHB metabolism may provide a probe of the physiological status of these complex microbial assemblies.

ACKNOWLEDGMENTS

We thank Loretta Ellias of the Department of Biological Science, Florida State University, for the *B. megaterium* and S. J. Morrison for the *E. coli*.

This study was supported by grant 04-7-158-44046 from the Office of Sea Grant, National Oceanographic and Atmospheric Administration, Department of Commerce, and by grant 76-19671 from the Biological Oceanography Program of the National Science Foundation.

We also thank Patrick Amoe of Eau Claire Memorial High School, Eau Claire, Wis., for the PHB determinations from Apalachicola Bay mud. He was supported by grant SMI-77-00768 from the National Secondary Science Training Program.

LITERATURE CITED

1. **Alper, R., and D. E. Lungren.** 1963. Properties of poly-β-hydroxybutyrate. I. General considerations concerning the naturally-occurring polymer. Biopolymers **1**:545–555.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
3. **Bobbie, R. J., S. J. Morrison, and D. C. White.** 1978. Effects of substrate biodegradability on the mass and activity of the associated estuarine microbiota. Appl. Environ. Microbiol. **35**:179–184.
4. **Bosshard-Heer, E., and R. Bachofen.** 1969. Synthese von Speicherstuffen aus Pyruvat durch *Rhodospirillum rubrum*. Arch. Mikrobiol. **65**:61–75.
5. **Burdon, K. L.** 1946. Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. J. Bacteriol. **52**:665–678.
6. **Carr, N. G.** 1966. The occurrence of poly-β-hydroxybutyrate in the blue-green alga *Chlorogloea fritschii*. Biochim. Biophys. Acta **120**:308–310.
7. **Crabtree, K., W. Boyle, E. McCoy, and G. A. Rohlich.** 1966. A mechanism of floc formation by *Zoogloea ramigera*. J. Water Pollut. Control Fed. **38**:1968–1980.
8. **Crabtree, K., E. McCoy, W. C. Boyle, and G. A. Rohlich.** 1965. Isolation, identification, and metabolic role of sudanophilic granules of *Zoogloea ramigera*. Appl. Microbiol. **13**:218–226.
9. **Davis, J. B.** 1964. Cellular lipids of a *Nocardia* grown on propane and *n*-butane. Appl. Microbiol. **12**:301–304.
10. **Dawes, E. A.** 1976. Endogenous metabolism and the survival of starved prokaryotes. Symp. Soc. Gen. Microbiol. **29**:19–53.
11. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **19**:135–278.
12. **Dienema, M. H.** 1972. Bacterial flocculation and production of poly-β-hydroxybutyrate. Appl. Microbiol. **24**:857–858.
13. **Doudoroff, M., and R. Y. Stanier.** 1959. Role of poly-β-hydroxy butyric acid in the assimilation of organic carbon by bacteria. Nature (London) **183**:1440–1442.
14. **Emeruwa, A. C., and R. Z. Hawirko.** 1973. Poly-β-hydroxybutyrate metabolism during growth and sporulation of *Clostridium botulinum*. J. Bacteriol. **116**:989–993.
15. **Gottschalk, G.** 1964. Die Biosynthese der poly-β-hydroxy Buttersäure durch Knallgasbakterien. II. Verwertung organischer Säuren. Arch. Mikrobiol. **47**:230–235.
16. **Jackson, F. A., and E. A. Dawes.** 1976. Regulation of the tricarboxylic acid cycle and poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. J. Gen. Microbiol. **97**:303–312.
17. **Kannan, L. V., and Z. Rehacek.** 1970. Formation of poly-β-hydroxybutyrate by Actinomycetes. Indian J. Biochem. **7**:126–129.
18. **King, J. D., and D. C. White.** 1977. Muramic acid as a measure of microbial biomass in estuarine and marine samples. Appl. Environ. Microbiol. **33**:777–783.
19. **King, J. D., D. C. White, and C. W. Taylor.** 1977. Use of lipid composition and metabolism to examine structure and activity of estuarine detrital microflora. Appl. Environ. Microbiol. **33**:1177–1183.
20. **Kominek, L. A., and H. O. Halvorson.** 1965. Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus*. J. Bacteriol. **90**:1251–1259.
21. **Kutty, M. R., L. V. Kannan, and Z. Rehacek.** 1969. Effect of phosphate on biosynthesis of antimycin A and production and utilization of poly-β-hydroxybutyrate by *Streptomyces antibioticus*. Indian J. Biochem. **6**:230–231.
22. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82**:33–36.
23. **Lundgren, D. G., R. Alper, C. Schnaitman, and R. H. Marchessault.** 1965. Characterization of poly-β-hydroxybutyrate extracted from different bacteria. J. Bacteriol. **89**:245–251.
24. **Macrae, P. M., and J. F. Wilkinson.** 1958. Poly-β-hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. J. Gen. Microbiol. **19**:210–222.
25. **Morrison, S. J., J. D. King, R. J. Bobbie, R. E. Bechtold, and D. C. White.** 1977. Evidence for microfloral succession on allochthonous plant litter in Apalachicola Bay, Florida, USA. Mar. Biol. **41**:229–240.
26. **Nakata, H. M.** 1966. Role of acetate in sporogenesis of *Bacillus cereus*. J. Bacteriol. **91**:784–788.
27. **Nuti, M. P., M. de Bertoldi, and A. A. Lepioi.** 1971. Influence of phenylacetic acid on poly-β-hydroxybutyrate (PHB) polymerization and cell elongation in *Azotobacter cholcoccum* Beij. Can. J. Microbiol. **18**:1257–1261.
28. **Pringsheim, E. G., and W. Wiessner.** 1963. Minimum requirements for heterotrophic growth and reserve substance in *Beggiatoa*. Nature (London) **197**:102.
29. **Schlegel, H. G., G. Gottschalk, and R. von Bartha.** 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (*Hydrogenomonas*). Nature (London) **191**:463–465.
30. **Schuster, E., and H. G. Schlegel.** 1967. Chemolithotrophes Wachstum von *Hydrogenomonas* L-16 im Chemostaten mit elektrolytischer Knallgaserzeugung. Arch. Mikrobiol. **68**:380–409.
31. **Senior, R. J., G. A. Beech, G. A. F. Ritchie, and E. A. Dawes.** 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. Bio-

chem. J. **128**:1193–1201.
32. **Short, S. A., D. C. White, and M. I. H. Aleem.** 1969. Phospholipid metabolism in *Ferrobacillus ferrooxidans*. J. Bacteriol. **99**:142–150.
33. **Sierra, G., and N. E. Gibbons.** 1962. Role of oxidation pathway of poly-β-hydroxybutyric acid in *Micrococcus halodenitrificans*. Can. J. Microbiol. **8**:255–268.
34. **Slepecky, R. A., and J. H. Law.** 1961. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. J. Bacteriol. **82**:37–42.
35. **Sobek, J. M., J. F. Charba, and W. N. Foust.** 1966. Endogenous metabolism of *Azotobacter agilis*. J. Bacteriol. **92**:687–695.
36. **Stanier, R. Y., M. Doudoroff, R. Kunisawa, and R. Contopoulou.** 1959. The role of organic substrates in bacterial photosynthesis. Proc. Natl. Acad. Sci. U.S.A. **45**:1246–1260.
37. **Stevenson, L. H., and M. D. Socolofsky.** 1966. Cyst formation and poly-β-hydroxybutyric acid accumulation in *Azotobacter*. J. Bacteriol. **91**:304–310.
38. **Stokes, J. L., and M. T. Powers.** 1967. Stimulation of poly-β-hydroxybutyrate. Oxidation of *Sphaerotilus discophorus* by manganese and magnesium. Arch. Mikrobiol. **59**:295–301.
39. **Uffen, R. L., C. Sybesma, and R. S. Wolfe.** 1971. Mutants of *Rhodospirillum rubrum* obtained after long-term anaerobic, dark growth. J. Bacteriol. **108**:1348–1356.
40. **Ward, A. C., and E. A. Dawes.** 1973. A disk assay for poly-β-hydroxybutyrate. Anal. Biochem. **52**:607–613.
41. **White, D. C.** 1971. The involvement of lipids in the modification of the bacterial membrane, p. 219–225. *In* A. Perez-Miravete and D. Pelaez (ed.), Recent advances in microbiology. Associacion Mexicana de Microbiologia, Mexico, D.F.
42. **White, D. C., R. J. Bobbie, J. S. Herron, J. D. King, and S. J. Morrison.** 1978. Biochemical measurements of microbial mass and activity from environmental samples. *In* Proc. Am. Soc. Test Mater. Symp., Native aquatic bacteria, enumeration, activity and ecology (Minneapolis, 25 June 1977).
43. **White, D. C., R. J. Bobbie, S. J. Morrison, D. K. Oosterhof, C. W. Taylor, and D. A. Meeter.** 1977. Determination of microbial activity of estuarine detritus by relative rates of lipid biosynthesis. Limnol. Oceanogr. **22**:1089–1099.
44. **White, D. C., and A. N. Tucker.** 1969. Phospholipid metabolism during changes in the proportions of membrane-bound respiratory pigments in *Haemophilus parainfluenzae*. J. Bacteriol. **97**:199–209.
45. **Wilkinson, J. F., and A. S. Munro.** 1967. The influence of growth limiting conditions on the synthesis of possible carbon and energy storage polymers of *Bacillus megaterium*. p. 173–185. *In* E. O. Powell, C. G. T. Evans, R. E. Strange, and D. W. Tempest (ed.), Microbial physiology and continuous culture. Proceedings of the 3rd International Symposium. Her Majesty's Stationery Office, London.
46. **Williamson, D. H., and J. F. Wilkinson.** 1958. The isolation and estimation of poly-β-hydroxybutyrate inclusions of Bacillus species. J. Gen. Microbiol. **19**:198–209.
47. **Zevenhuizen, L. P. T. M., and A. G. Ebbink.** 1974. Interrelation between glycogen poly-β-hydroxybutyric acid and lipids during accumulation and subsequent utilization in a Pseudomonas. Antonie van Leeuwenhoek J. Microbiol. Serol. **40**:103–120.