# Metabolism of Poly-β-hydroxybutyrate. I. Purification, Composition, and Properties of Native Poly-β-hydroxybutyrate Granules from *Bacillus megaterium**

R. Griebel, Z. Smith, and J. M. Merrick

ABSTRACT: Native poly-β-hydroxybutyrate granules from *Bacillus megaterium* have been purified and their composition and properties have been studied. The granules are composed of approximately 98% poly-β-hydroxybutyrate, 2% protein, and small amounts of lipid. The lipid components comprise an unidentified acetone-soluble compound and a single phospholipid identified as phosphatidic acid. The purified granules are surrounded by a membrane coat and possess similar properties to those previously reported for crude preparations.

The poly-β-hydroxybutyrate synthetase remains firmly bound to the granules throughout the purification. The $K_M$ for the synthetase is $9.25 \times 10^{-5}$ M and its pH optimum is 7.5. The synthetase is inhibited by low levels of *N*-ethylmaleimide and *p*-mercuribenzoate.

Many diverse bacterial species accumulate PHB,[1] the polymeric ester of D-(−)-β-hydroxybutyrate, as their principal lipid reserve. The polymer occurs as discrete granules in the cell, and may constitute under certain conditions more than 50% of the dry weight of the cell. Its enzymatic synthesis and degradation have been studied in cell-free preparations of *Rhodospirillum rubrum* and *Bacillus megaterium* (Merrick and Doudoroff, 1961, 1964; Merrick and Yu, 1966). The enzymatic depolymerization of PHB consists of at least two soluble factors (activator and depolymerase) whose successive action results in the breakdown of PHB granules to soluble products. An extremely labile particulate component is also required and is associated with PHB granules. Evidence has been presented which suggests that this factor may be contained in the membranous coat which surrounds the granule (Lundgren *et al.*, 1964; Merrick, 1965; Merrick *et al.*, 1965).

The enzymatic synthesis of PHB has been less extensively studied than its depolymerization. It has however been demonstrated that isolated PHB granules from *B. megaterium* or *R. rubrum* contain an enzyme(s) (PHB synthetase) which will catalyze the polymerization of β-hydroxybutyrate from D-(−)-β-hydroxybutyryl-CoA. Presumably, the synthetase is also associated with the membrane coat of the granules; however evidence is available which suggests that the synthetase and the labile particulate factor involved in PHB depolymerization are different components of the granule (Merrick and Doudoroff, 1964; Merrick, 1965).

The above studies have suggested that PHB granules are complex subcellular particles that possess bound factors which contribute to both the synthesis and degradation of PHB in the granule. Earlier studies of the chemical composition of PHB inclusions have been carried out with preparations isolated by procedures which destroy their organization as well as the active factors (Williamson and Wilkinson, 1958). The composition of native PHB granules with the properties described above was however unknown. In this communication, we describe fractionation procedures that crude PHB granules have been subjected to in order to remove loosely bound substances. These procedures are relatively mild and result in the isolation of intact native PHB granules. Examination of these granules in the electron microscope revealed that they were free from other contaminating particulate cellular constituents. The composition of these granules as well as their properties are described. In addition the properties of the bound PHB synthetase have been further studied and the results are presented.

## Experimental Section

*Materials. B. megaterium* KM was grown in a medium containing 0.3% glucose and 0.05 M sodium acetate as described by Macrae and Wilkinson (1958). The cells were harvested near the end of exponential growth and stored as a cell paste at −20° until needed.

D-(−)-β-Hydroxybutyryl-CoA was prepared by the mixed-anhydride method of Wieland and Rueff (1953). Radioactive D-(−)-β-hydroxybutyrate was obtained as described by Merrick and Doudoroff (1961). Phospholipid chromatographic standards were purchased from Applied Science Laboratories and were also kindly provided by Drs. W. E. M. Lands, H. Goldfine, and M. Colodzin. Dextran-500 was purchased from Pharmacia Co. and polyethylene glycol (Carbowax 6000) was obtained from Union Carbide Corp. All other chemicals

* From the Biological Research Laboratories, Department of Bacteriology and Botany, and Biochemistry Committee, Syracuse University, Syracuse, New York 13210. *Received June 7, 1968.* This investigation was supported by Research Grant AI-07176 from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PHB, poly-β-hydroxybutyrate; CoA, coenzyme A.

were commercial preparations. All solvents were redistilled. Peroxide-free ether was obtained by passage of redistilled ether through a column of alumina (Dasler and Bauer, 1946). Acetone was refluxed with potassium permanganate, dried over anhydrous potassium carbonate, and fractionally distilled (Vogel, 1954).

*Methods.* CHROMATOGRAPHY. Neutral lipids and phospholipids were analyzed by thin-layer chromatography using Eastman chromatogram sheets (Type K301R2). The sheets were activated at 100° for 1 hr and stored in a desiccator until used. Developing solvent systems used for neutral lipids were (A) ether–glacial acetic acid–*n*-hexane (25:2:73, v/v) (Krell and Hashim, 1963) and (B) heptane–ethyl acetate (90:10, v/v) (Thorne and Kodicek, 1966). Chromatograms were visualized using $I_2$ vapors or 0.2% 2,7-dichlorofluorescein. Thin-layer chromatography of phospholipids was carried out in (C) chloroform–methanol–glacial acetic acid–water (70:10:7:3, v/v), a modification of the system described by Skipski *et al.* (1962). Phospholipids were also subjected to chromatography on silicic acid impregnated paper prepared according to Marinetti (1962). In this case the solvent system employed was (D) diisobutyl ketone–glacial acetic acid–water (40:20:3, v/v). Chromatograms were visualized with Rhodamine 6 G (Marinetti, 1962), ammonium molybdate (Bandurski and Axelrod, 1951), and iodine vapors.

EXTRACTION OF LIPIDS FROM PURIFIED NATIVE PHB GRANULES. Purified PHB granules were lyophilized following exhaustive dialysis against 0.01 M KCl. The granules (150–200 mg) were extracted three times with 5-ml portions of acetone at room temperature with stirring for 20 min. After centrifugation, the acetone extracts were combined, filtered, and evaporated to dryness under nitrogen. The residue was dissolved in acetone and refiltered to remove traces of salt. The acetone-extracted granules were subjected to extraction (three times) with 5-ml portions of alcohol–ether (3:1, v/v) at 35° for 20 min each time. After centrifugation, the combined extracts were filtered and concentrated to approximately 0.5 ml under $N_2$. Chloroform (10.0 ml) was added and the sample was again evaporated to approximately 0.5 ml under nitrogen. This process was repeated a second time. The remaining PHB granule residue was further extracted in a Soxhlet apparatus for 8 hr with ether. The ether extract was concentrated *in vacuo* at 35° and the residue was dissolved in chloroform. Finally the alcohol–ether and ether extracts were combined and filtered. All filtrations were performed on Whatman No. 41 filter paper that had been preextracted with acetone, alcohol–ether, and finally chloroform.

ENZYME ASSAYS. PHB synthetase was measured by a modification of the procedure previously described by Merrick and Doudoroff (1961). The reaction mixture contained 2.5 μmoles of Tris-HCl buffer (pH 7.5), 0.5 μmole of $MgCl_2$, 0.5 μmole of 2-mercaptoethanol, 10 μg of albumin, 9.45 mμmoles of D-(−)-β-hydroxybutyryl-$^{14}$C-CoA (specific activity $3.09 \times 10^6$ cpm per μmole), and native PHB granules which contain the synthetase. Final volume was 0.05 ml. After incubation for 3 min at 30° the reaction was terminated by the addition of 1.0 ml of ethanol followed by 4 mg of carrier purified PHB (Merrick and Yu, 1966). After centrifugation the polymer was washed successively with 1.5-ml portions of water, 10% ethanol, and acetone. Controls included tubes without enzyme and reactions terminated at zero time. Finally the pellet was dissolved in $CHCl_3$ and suitable aliquots were analyzed for radioactivity in a Nuclear-Chicago gas-flow counter. One unit of enzyme activity was defined as that amount of enzyme which catalyzes the transfer of 1 μmole of β-hydroxybutyrate-$^{14}$C/min to PHB under the conditions described above. The preparation of activator and depolymerase as well as measurement of depolymerization of PHB was carried out as described by Merrick and Doudoroff (1964), unless otherwise indicated.

OTHER ASSAYS. Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. The protein content of PHB granules was determined after solubilization of the granules in 0.14 N NaOH at 100° for 20 min. Total β-hydroxybutyrate was determined by the method of Slepecky and Law (1960). Organic and inorganic phosphorus was analyzed according to the procedure of Bartlett (1959). Carbohydrate was measured according to the anthrone procedure described by Radin *et al.* (1955). Aminophosphatides were detected with ninhydrin as described by Marinetti (1962). Dry weight determinations of extracted lipids were performed by evaporating samples to constant weight at 80°. Total dry weight of PHB granules was determined after drying samples to constant weight at 100°.

Specimens for electron microscopy were prepared by the carbon replica technique as previously described (Merrick *et al.*, 1965).

## Results

*Purification of B. megaterium PHB Granules.* PREPARATION OF CELL-FREE EXTRACTS. *B. megaterium* cells (20 g wet weight) were suspended in 60 ml of 0.05 M Tris-HCl buffer (pH 8.0)–$1.67 \times 10^{-2}$ M $MgCl_2$. Lysozyme (66 mg) and deoxyribonuclease (0.7 mg) were added and the suspension was allowed to incubate for 30 min at room temperature. After cooling to 0° the suspension in 60-ml portions was subjected to sonic oscillation for 3 min with a Bronwill Biosonic probe, Model Biosonik II. The PHB granules were separated from the crude extract by layering the suspension on glycerol,[2] followed by centrifugation in a swinging-bucket rotor (International Clinical Model centrifuge) at 1600*g* for 15 min at 4°. After removal of the supernatant fluid, the PHB granules which collect at the interface were resuspended in 60.0 ml of 0.05 M Tris-HCl (pH 8.0). Granules prepared in the above manner constitute crude PHB granules.

[2] PHB granules are readily inactivated as a substrate for the PHB depolymerizing enzymes by repeated centrifugation. However, as described previously (Merrick and Doudoroff, 1964), inactivation by centrifugation could be minimized by layering the suspension on a cushion of glycerol introduced at the bottom of the centrifuge tube. All centrifugations of PHB granules were carried out in this manner.

TABLE I: Purification of Native PHB Granules from *B. megaterium*.

| Step | Vol (ml) | PHB (mg/ml) | Protein (mg/100 mg of PHB) | Phosphate ($\mu$moles/ 100 mg of PHB) | % Recov of PHB | PHB Synthetase | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Units/ml | Sp Act. (units/mg of protein) | % Recov |
| Crude PHB granules | 62.0 | 8.4 | 6.78 | 7.80 | 100 | 4.94 | 8.7 | 100 |
| Differential centrifugation | 14.3 | 26.4 | 2.70 | 1.39 | 72.5 | 12.0 | 16.8 | 56 |
| Polymer two-phase system | 24.4 | 13.3 | 2.49 | 0.69 | 62.5 | 4.3 | 12.9 | 34 |
| Density gradient centrifugation | 15.5 | 16.6 | 2.33 | 0.56 | 49.5 | 5.9 | 15.2 | 30 |

DIFFERENTIAL CENTRIFUGATION. Differential centrifugation was carried out first at 1000*g*, then at 650*g*, and finally at 450*g* for 45 min, and the granules were collected from the interface each time. Before each centrifugation the granules were resuspended in 60 ml of 0.05 M Tris-HCl (pH 8.0). After the last centrifugation, the granules were resuspended in 10.0 ml of 0.05 M Tris-HCl (pH 8.0) and dialyzed for 15 hr against 2.0 l. of 0.02 M Tris-HCl (pH 8.0)–5 $\times$ $10^{-4}$ M EDTA, followed by dialysis against 2.0 l. of 0.02 M Tris-HCl (pH 8.0) for 2 hr. After dialysis the supernatant fluid was removed from the settled granules and the granules were resuspended in 0.02 M Tris-HCl (pH 8.0). Final volume was 14.3 ml.

POLYMER TWO-PHASE SYSTEM. The granule suspension (diluted with 0.02 M Tris-HCl (pH 8.0) so that 20 ml contains between 300 and 500 mg of PHB) was subjected to sonic oscillation for 1 min before being introduced into the polymer two-phase system described by Hofsten and Baird (1962). The system contained 5.0% w/w Dextran-500 and 3.5% w/w polyethylene glycol and was buffered with 0.02 M Tris-HCl (pH 8.0); 1 ml of the two-phase system was used for each 10 mg of PHB to be purified. The system was gently inverted 20–30 times to ensure thorough mixing, and then allowed to stand for 30–60 min at 4°. Under these conditions the PHB granules partition to the lower dextran-rich phase, while other particulate matter partitions to the top polyethylene glycol rich phase. The upper phase was removed and was replaced with an equal volume of fresh upper phase. The system was again mixed by gentle inversion and the two phases were allowed to separate a second time. This washing procedure with fresh upper phase was repeated a third time.

In order to separate the lower phase from the granules, it was diluted fourfold with 0.02 M Tris-HCl (pH 8.0) and the granules were allowed to settle. After removal of the supernatant fluid, the granules were resuspended in a volume of buffer equal to that removed and the process was repeated. Final volume was 24.4 ml.

DENSITY GRADIENT CENTRIFUGATION OF PHB GRANULES. Glycerol gradients were prepared by layering 5.0 ml each of 10.5, 10.0, 9.5, and 9.0 M glycerol followed by equilibration for at least 48 hr at room temperature. The granules were diluted with 0.02 M Tris-HCl (pH 8.0) to 45.0 ml. The suspension (15 ml) containing approximately 100 mg of PHB was layered on each of three glycerol gradients and centrifugation was carried out at 90,000*g* for 75 min in a Spinco Model L ultracentrifuge at 0°. PHB granules, which band between 9.0 and 9.5 M glycerol, were removed and dialyzed exhaustively against 0.02 M Tris-HCl (pH 8.0) to remove the glycerol. The supernatant fluid is removed from the settled granules and the granules are resuspended in 0.02 M Tris-HCl (pH 8.0). Final volume was 15.5 ml. PHB granules, prepared by the procedures described above, constitute purified native PHB granules. A summary of a typical purification is presented in Table I. The purified PHB granules were obtained in approximately 50% yield. As indicated, the granules contain both protein and phosphate. The purified granules were stored at 4° in a dialysis sac surrounded by 0.02 M Tris-HCl buffer (pH 8.0).

*Chemical Composition.* The chemical composition of purified native PHB granules is seen in Table II. PHB constitutes 97–98% of the dry weight of the granule. The remainder is principally composed of protein and lipid. As indicated in Table I native granules also contain phosphate. While insignificant amounts of organic

TABLE II: Chemical Composition of Purified PHB Granules.

| | Dry Wt (%) |
|---|---|
| PHB | 97.7 |
| Protein | 1.87 |
| Lipid | |
| Acetone extractable | 0.21 |
| Alcohol–ether and ether extractable | 0.25 |

phosphate were extracted with acetone, approximately 30–50% was found routinely in the alcohol–ether and ether extract. Continuous extraction of alcohol–ether and ether-extracted granules with $CHCl_3$ and $CHCl_3$–methanol (2:1, v/v) failed to solubilize any additional organic phosphate and suggests the presence of nonlipid phosphate as a component of the granules. Chromatographic analysis revealed that a single phospholipid was present in the alcohol–ether extract. On thin-layer chromatography this component migrated with an $R_F$ of 0.98 and on silicic acid impregnated paper the component migrated with an $R_F$ of 0.80. In both cases, migration was identical with that of known phosphatidic acid and differed from cardiolipin or phosphatidylglycerol. No ninhydrin-positive material was present in the alcohol–ether and ether extract. The phospholipid was deacylated by mild alkaline hydrolysis according to the procedure of Dawson (1955) as modified by Benson and Strickland (1960). After neutralization with Dowex 50 ($H^+$) resin the phosphate ester was subjected to two-dimensional chromatography on Whatman No. 4 paper in phenol–water (100:38, v/v) and butanol–propionic acid–water (142:71:100, v/v). The chromatograms were developed with ferric chloride–sulfosalicylic acid (Wade and Morgan, 1953). Standard phosphatidic acid was treated in a similar manner. Both the standard and the phospholipid present in the alcohol–ether and ether extract gave rise to a phosphate ester whose migration was identical with known glycerol phosphate. The above results, therefore, strongly suggest that the phospholipid in the alcohol–ether and ether extract is phosphatidic acid.

The acetone-extracted lipid exhibited a single component when chromatographed in solvent systems A and B. The $R_F$ values varied from 0.50 to 0.55 in solvent system A and from 0.40 to 0.45 in solvent system B. The following substances migrated differently in the above solvent systems: monopalmitin, dipalmitin, tripalmitin, triolein, oleic acid, stearic acid, and β-hydroxybutyric acid. The lipid is stable to alkaline hydrolysis in 0.1 N methanolic KOH for 1 hr at 35°. Hydrolysis in 0.1 N HCl at 100° for 10 min resulted in the formation of two additional components with $R_F$ values of 0.11 and 0.32 in solvent system B. The substance is soluble in chloroform, methanol, butanol, and ether. It contains no phosphate, is not detected with ninhydrin spray, and gives a negative anthrone test. No further studies were carried out with this component.

*Properties of Purified Native PHB Granules.* The properties of the purified granules are similar to those previously reported for crude PHB granules (Merrick and Doudoroff, 1964). Thus as seen in Table III, depolymerization requires either activator or trypsin treatment of the granules before depolymerase, the enzyme which presumably carries out the hydrolysis,[3] can attack the polymer. The purified granules are still sensitive to a variety of different treatments. Thus heating the granules for 5 min at 100° destroys over 50% of the substrate; a 30% loss was obtained after freezing and thawing five times while over 40% was lost after four repeated centrifugations. Extensive digestion with trypsin according to procedures previously described by Merrick *et al.* (1965) completely destroys the capacity of the granules to act as a substrate.

TABLE III: Hydrolysis of Purified PHB Granule.[a]

| Additions to PHB Granules | | μmoles of Acid Produced in 20 min |
|---|---|---|
| Before Preincubation | After Preincubation | |
| None | None | 0.0 |
| None | Depolymerase | 0.0 |
| Activator | None | 0.0 |
| Activator | Depolymerase | 5.0 |
| Trypsin | None | 0.0 |
| Trypsin | Depolymerase | 4.4 |

[a] Reactions were carried out in a Radiometer pH-Stat and a constant pH of 8.3 was maintained by the addition of potassium hydroxide. Concentration of purified PHB granules was based on total β-hydroxybutyrate content and corresponded to 12 μmoles of the monomer. Preincubation of PHB granules alone or in the presence of activator (65 μg) or trypsin (2.5 μg) was carried out for 20 min. Reactions where indicated were initiated with depolymerase (2.0 μg). Initial volume of the reaction mixture was 1.3 ml; temperature was 30°.

In addition to the labile component associated with hydrolysis of the polymer, crude PHB granules also contain the PHB synthetase (Merrick and Doudoroff, 1961). Granules purified by the procedures described above are still associated with the PHB synthetase (Table I). Throughout the purification there appears to be a greater loss of enzyme than of polymer. Although these data suggest that some of the enzyme may be removed by the purification procedure, attempts to recover additional synthetase activity proved unsuccessful. Thus, for example, the supernatant fluid after the differential centrifugation step was again centrifuged to obtain the residual granules. Such granules alone or in combination with the supernatant fluid could only account for 3% of the original enzymatic activity. Furthermore, the supernatant fluid and the residual granules, alone or in combination, did not stimulate the synthesis of PHB by active preparations obtained by differential centrifugation. All studies of the PHB synthetase to be described were carried out with preparations of granules obtained after density gradient centrifugation. The effect of incubation time, protein concentration, and pH on the reaction velocity are illustrated in Figures 1 and 2. The pH optimum was found to be 7.5. The $K_M$ calculated

[3] Recently, support for the conclusion that the depolymerase fraction carries out the actual hydrolysis of PHB has been obtained in studies with the trimeric ester of β-hydroxybutyrate. The depolymerase hydrolyzes trimer to monomer and dimer and does not require the presence of either the activator or trypsin (T. Schwartz and J. M. Merrick, unpublished observations).

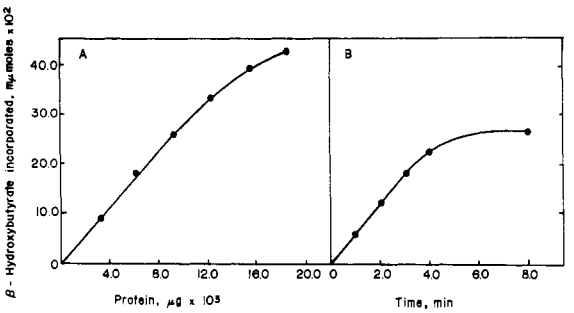


FIGURE 1: Effect of enzyme concentration (A) and incubation time (B) on PHB synthetase activity. Conditions as in standard assay. In B, reaction mixtures contained $6.2 \times 10^{-3}$ μg of protein.

according to Lineweaver and Burk (1934) was $9.25 \times 10^{-5}$ M (Figure 3).

Table IV shows the requirements of the reaction. The addition of $MgCl_2$, albumin, or 2-mercaptoethanol stimulated the enzymatic activity from about two- to fourfold. If all three were present simultaneously the reaction was stimulated to about fivefold.

The results of studies in which the PHB synthetase was incubated with various sulfhydryl inhibitors are shown in Table V. Both *p*-mercuribenzoate or *N*-ethylmaleimide strongly inhibited the reaction, suggesting that the PHB synthetase is a sulfhydryl enzyme. Iodoacetamide was somewhat less effective as an inhibitor. The purified native PHB granules examined under the electron microscope exhibited similar morphology to those previously reported and appeared to

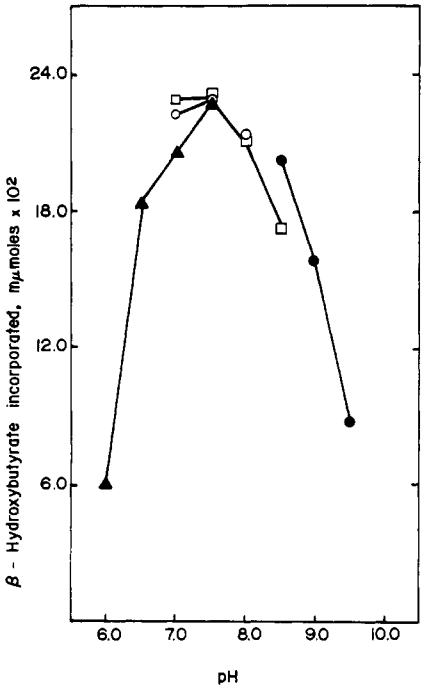


FIGURE 2: Effect of pH on PHB synthetase activity. Conditions as in standard assay but with the following buffers: (▲—▲) imidazole-HCl; (○—○) potassium phosphate; (□—□) Tris-HCl; (●—●) glycine–NaOH. Reaction mixtures contained $1.2 \times 10^{-2}$ μg of protein.

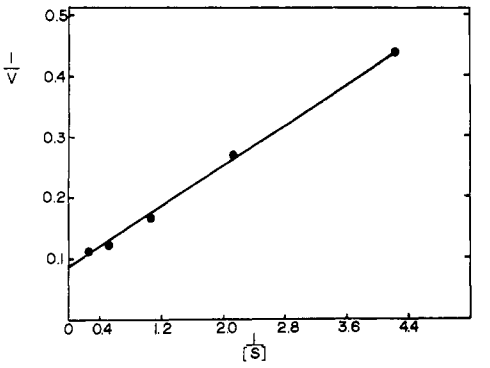


FIGURE 3: Effect of substrate concentration on PHB synthetase activity. Conditions as in standard assay. $V$ is mμmoles of β-hydroxybutyrate incorporated per min $\times 10^2$ and $S$ is in $10^{-4}$ M. Reaction mixtures contained $1.1 \times 10^{-2}$ μg of protein.

be free of any other contaminating particulate cellular constituents (Merrick *et al.*, 1965).

## Discussion

The lability of native PHB granules to a variety of agents has previously hindered their purification. However the procedures described in this communication are relatively mild and rapid and give rise to preparations that appear to be free of any other contaminating cellular constituents as visualized by electron microscopy. Purified native PHB granules possess properties similar to those previously reported for crude preparations. Characteristically, inactivated granules demonstrate the discrete membrane which encases the granule. Although it was initially speculated that the granule membrane may be derived from the cell membrane, extensive studies by Ellar *et al.* (1968) have failed to find evidence of a typical unit membrane surrounding the granule. These and other studies have suggested that the PHB membrane is structurally less complex

TABLE IV: Requirements for PHB Synthetase Activity.[a]

| Additions | β-Hydroxybutyrate-$^{14}$C Incorp (mμmoles $\times 10^2$) |
|---|---|
| None | 5.0 |
| $MgCl_2$ | 10.8 |
| Albumin | 18.8 |
| 2-Mercaptoethanol | 15.0 |
| Albumin, 2-mercaptoethanol, $MgCl_2$ | 24.0 |

[a] The complete system contained in 0.05 ml: Tris-HCl (pH 7.5), 2.5 μmoles; D-(−)-β-hydroxybutyryl-CoA, 9.45 mμmoles (specific activity, $3.09 \times 10^6$ cpm/μmole); and PHB synthetase, 0.009 μg. Albumin (10 μg), $MgCl_2$ (0.5 μmole), and 2-mercaptoethanol (0.5 μmole) were added as indicated. Assays were carried out as described in the Experimental Section.

TABLE V: Effect of Sulfhydryl Inhibitors on PHB Synthetase.[a]

| Inhibitor | Concn (M) | Inhibn (%) |
|---|---|---|
| Iodoacetamide | $4 \times 10^{-3}$ | 44.1 |
| | $4 \times 10^{-2}$ | 100 |
| *N*-Ethylmaleimide | $2 \times 10^{-4}$ | 81.4 |
| | $4 \times 10^{-2}$ | 100 |
| *p*-Mercuribenzoate | $4 \times 10^{-6}$ | 69.7 |
| | $2 \times 10^{-5}$ | 100 |

[a] Assays were carried out as described in the Experimental Section. PHB synthetase ($4.3 \times 10^{-2}$ μg) was preincubated for 10 min at 30° with the indicated concentration of sulfhydryl inhibitor. Reactions were initiated by the addition of substrate.

than the cell membrane. Chemical analysis has revealed that purified granules contain less than 2% protein. Lipid constituents consist of a single unidentified acetone-soluble lipid and a single phospholipid, phosphatidic acid. These substances presumably constitute the membrane coat which surrounds the core PHB.

The relationship of the structure of the PHB granule to its synthesis and degradation is still obscure. Most likely the PHB synthetase and the labile factor involved in depolymerization are associated with the membrane. The membrane coat may also be involved in regulation of PHB synthesis and degradation as well as contributing to the morphological integrity of the granule by maintaining a stable colloidal particle as suggested by Ellar *et al.* (1968). These workers have recently investigated the structural organization of native PHB granules and have shown that the granule is composed of 100–150-Å fibrils involving extended polymeric chains. Each granule on an average contains at least 10,000 chain molecules. A model which explains the observed morphology proposes that polymer-synthesizing enzymes aggregate into a micellar form and that PHB synthesis occurs within this protein covering, giving rise to PHB fibrils parallel to each other, compacted in the spherical granule. The surface coat, therefore, would be primarily composed of polymer-synthesizing molecules.

Physiological studies have indicated that PHB functions as an endogenous reserve analogous to that of starch or glycogen. However the metabolism of this substance raises a pertinent, biochemically fundamental problem of how an extremely hydrophobic molecule is rapidly synthesized and easily degraded by reactions which take place predominantly in an aqueous environment. It seems reasonable to assume that the surface coat of the granule must play an important role in this regard. Further studies are currently in progress in an attempt to elucidate the functional relationship between the protein, lipid, and PHB of the granule.

Acknowledgment

We wish to express our appreciation to Dr. D. G. Lundgren for carrying out the electron microscopy, and to Miss Elizabeth Smola for her assistance in the identification of phosphatidic acid.

References

Bandurski, R. S., and Axelrod, B. (1951), *J. Biol. Chem. 193*, 405.
Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Benson, A. A., and Strickland, E. H. (1960), *Biochim. Biophys. Acta 41*, 328.
Dasler, W., and Bauer, C. D. (1946), *Ind. Eng. Chem. Anal. Ed. 18*, 52.
Dawson, R. M. C. (1955), *Biochem. J. 59*, 5.
Ellar, D., Lundgren, D. G., Okamura K., and Marchessault, R. H. (1968), *J. Mol. Biol.* (in press).
Hofsten, B. V., and Baird, G. D. (1962), *Biotechnol. Bioeng. 4*, 403.
Krell, K., and Hashim, S. A. (1963), *J. Lipid Res. 4*, 407.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Lundgren, D. G., Pfister, R. M., and Merrick, J. M. (1964), *J. Gen. Microbiol. 34*, 441.
Macrae, R. M., and Wilkinson, J. F. (1958), *J. Gen. Microbiol. 19*, 210.
Marinetti, G. V. (1962), *J. Lipid Res. 3*, 1.
Merrick, J. M. (1965), *J. Bacteriol. 90*, 965.
Merrick, J. M., and Doudoroff, M. (1961), *Nature 189*, 890.
Merrick, J. M., and Doudoroff, M. (1964), *J. Bacteriol. 88*, 60.
Merrick, J. M., Lundgren, D. G., and Pfister, R. M. (1965), *J. Bacteriol. 89*, 234.
Merrick, J. M., and Yu, C. I. (1966), *Biochemistry 5*, 3563.
Radin, N. S., Lavin, F. B., and Brown, J. R. (1955), *J. Biol. Chem. 217*, 789.
Skipski, V. P., Peterson, R. F., and Barclay, M. (1962), *J. Lipid Res. 3*, 467.
Slepecky, R. A., and Law, J. H. (1960), *Anal. Chem. 32*, 1967.
Thorne, K. J. I., and Kodicek, E. (1966), *Biochem. J. 99*, 123.
Vogel, A. I. (1954), A Textbook of Practical Organic Chemistry, London, Longmans, Green, p 170.
Wade, H. E., and Morgan, D. M. (1953), *Nature 171*, 529.
Wieland, T., and Rueff, L. (1953), *Angew. Chem. 65*, 186.
Williamson, D. H., and Wilkinson, J. I. (1958), *J. Gen. Microbiol. 19*, 198.