Structure of Poly- β -hydroxybutyric Acid Granules

BY D. G. LUNDGREN AND R. M. PFISTER

Department of Bacteriology and Botany, Syracuse University, Syracuse, New York, U.S.A.

AND J. M. MERRICK

Department of Biochemistry, University of Buffalo, Buffalo, New York, U.S.A.

(Received 6 August 1963)

SUMMARY

A membrane surrounding poly- β -hydroxybutyrate (PHB) granules isolated from both *Bacillus cereus* and *B. megaterium* has been demonstrated by the carbon-replica technique and electron microscopy. Some general features and properties of both the membrane and PHB granules are discussed.

INTRODUCTION

The polymeric ester, poly- β -hydroxybutyric acid (PHB) has been studied recently as regards its role as a storage product (Doudoroff & Stanier, 1959; Macrae & Wilkinson, 1958), its enzymic synthesis and breakdown (Merrick & Doudoroff, 1961; Merrick, Delafield & Doudoroff, 1962), its formation in bacteria (Schlegel, Gottschalk & von Barth, 1961; Sierra & Gibbons, 1962), its assay (Slepecky & Law, 1960), and its chemical and physical properties (Williamson & Wilkinson, 1958). The experimental work pertinent to PHB is expanding rapidly and the reader is referred to the interesting paper of Schlegel & Gottschalk (1962) for more information on the distribution of PHB, its function and biochemistry. The work reported here is the result of a collaborative effort by the authors from the laboratories cited. The likely presence of an envelope or membrane around PHB granules was independently uncovered while studying different properties of PHB. At Syracuse, studies were made of the crystalline structure of PHB isolated from both *Bacillus cereus* and a *Rhizobium* species (Alper, Marchessault & Lundgren, 1962; Alper, Lundgren & Marchessault, 1963; Alper, Lundgren, Marchessault & Cote, 1963; Hester, 1963), as well as an investigation of the accumulation of PHB in an auxotrophic mutant of *B. cereus* grown with either methionine or cyst(e)ine added to the minimal medium (Lundgren & Bott, 1963). In the laboratories at Buffalo, the enzymic depolymerization of PHB granules isolated from *B. megaterium* has been examined (Merrick *et al.* 1962).

METHODS

Preparation of PHB granules for electron microscopy

Bacillus cereus. Initially PHB granules examined with electron optics were those isolated from 69 hr *B. cereus* c-1 organisms. The complete culture system for this auxotrophic mutant of *B. cereus* ATCC 4342 has been described (Lundgren & Bott, 1963) and both stained and phase-contrast microphotographs of cells containing

polymer were shown. Bacilli containing PHB were centrifuged from 10 ml. of the 69 hr old culture, washed 3 times with distilled water, and subjected to sonic treatment in distilled water for 10 min. at an intensity equivalent to a dial setting '7' with a 20 keyc. Branson Sonifier (Heat Systems Co., 777 Northern Blvd., Great Neck, Long Island, N.Y.). After sonic treatment the crude extract was centrifuged at 2700 *g* for 15 min. The particulate matter was suspended in 10 ml. distilled water and when examined under phase optics showed individual, slightly refractile granules.

Bacillus megaterium. The PHB granules from *B. megaterium* KM were isolated as follows. The organism was grown as outlined by Merrick & Doudoroff (1961). Five g. bacilli (wet wt.; harvested at the end of exponential growth) were suspended in 30 ml. 0.05 M-potassium phosphate buffer (pH 7.0); 33 mg. lysozyme; 0.5 ml. M-MgCl₂ and 0.35 mg. deoxyribonuclease (Worthington Biochemical Corporation, Freehold, N.J.) were added and the mixture incubated for 30 min. The lysate was subjected to ultrasonic treatment with an ultrasonic probe for 2 min. to liberate the PHB granules from the cell membranes. The granules were separated from the crude extract by layering the lysate on glycerol followed by centrifugation in a swinging bucket rotor at 9000 *g* for 20 min. The supernatant fluid was then discarded and the PHB granules which collected on the surface of the glycerol were removed and resuspended in 30 ml. 0.05 M-tris-HCl buffer (pH 8.0). The centrifugation on glycerol was twice repeated, the final preparation of granules suspended in 5 ml. tris buffer system and then dialysed for 24 hr against 0.02 M-tris-HCl buffer (pH 8.0). We have referred to these granules as 'native' polymer granules for they were isolated by procedures less drastic than those used on *B. cereus*. The 'native' granules are readily susceptible to hydrolysis by cell-free extracts of *Rhodospirillum rubrum* organisms that have depleted their own polymer stores. However, these granules, when treated with acetone, ethanol, hypochlorite or heat, will no longer serve as a substrate for the depolymerizing system (Merrick *et al.* 1962). In some experiments the 'native' granules were also subjected to sonic oscillation for various periods of time or were treated with sodium lauryl sulphate.

Specimens for electron microscopy were prepared using the carbon replica techniques of Bradley & Williams (1957). Electron micrographs were taken with an RCA EMU 2D Electron Microscope, with a 50 or 25 μ objective aperture.

RESULTS

Plate 1, figs. 1 and 2, show electron micrographs of a germanium-shadowed carbon replica of PHB granules isolated from *Bacillus cereus* c-1. On many of the granules, fragments of a delicate skin-like structure can be seen. This membranous material, when intact, is apparently wrapped around the granules. This figure also shows the typical surface differences seen in all preparations examined. Some of the granules are smooth in appearance, while others are rough and irregular. These surface differences may, however, reflect the preparative procedures for electron microscope analysis. These differences were readily seen in all specimens examined. Some of the granules appeared to coalesce, a feature which is more demonstrable with granules having intact membrane covers. This property is presumably a result of the drying of the specimen, since synthetic polystyrene pellets coalesced under similar conditions. Differences in the shape and size of the PHB granules are readily

observable in Pl. 1, fig. 2. Although the majority of the granules appeared spherical, some rod-like structures were occasionally seen. These structures also have smooth or rough surfaces and are encased in a membrane. The rod-like granule which possessed the smooth surface appeared to have the membrane coat still intact. PHB granules vary in diameter from about 0.2 to 1.1 μ ; there were no noticeable differences in granules isolated from the two bacilli.

Plate 1, fig. 3, is a germanium-shadowed carbon replica of *Bacillus cereus* 4342 spores which is shown for comparative purposes to indicate the similarities and differences between spores and PHB granules. The ridged spores are similar to the *B. cereus* spores shown in the electron micrographs of Bradley & Williams (1957).

Germanium-shadowed carbon replicas of PHB granules isolated from *Bacillus megaterium* are shown in Plate 2. In Pl. 2, figs. 5–8, 'native' granules which were subjected to sonic treatment for 5 min. are shown with broken membranes surrounding the granules. Granules shown in Pl. 2, fig. 5, show the irregularity of the rough-surface granules. Native granules completely enclosed in membranes are seen in Pl. 2, fig. 6. A membrane wrinkle is seen on top of the large PHB granule and the membrane appears to be continuous with the other granules. Native granules shadowed with germanium at an angle of 45° are shown in Pl. 2, fig. 7; the polymer was electron dense with a less dense membrane around it. The other polymer granules in this preparation are clumped together. This is a feature frequently noted in many of the preparations.

Plate 2, fig. 8 to Pl. 3, fig. 11, show electron micrographs of carbon replicas of different PHB preparations from *Bacillus megaterium*. Plate 2, fig. 8, shows the highly dense peripheral portion of a membrane which appears to have more than one layer and which extends to the neighbouring granules as well. A small protrusion is also shown, extending from one of the larger granules. This may represent the early formation of another PHB granule encased within the same membrane. The extraneous material seen as part of the background represents cell debris carried over with the granules during the isolation procedure. Plate 3, fig. 9, shows a carbon replica of a *B. megaterium* with a flagellum and with large PHB granules deposited in (or on) the organism. The granules are still contained within the organism or are at a state just prior to their release after the cell wall was ruptured by sonic treatment and lysozyme. Plate 3, fig. 10, shows intact and enzymically active granules. When such granules were treated with 0.1 % sodium lauryl sulphate for 24 hr with shaking, isolated structures were seen that appeared to be membrane coats (Pl. 3, fig. 11). These were circular bodies with raised edges which were readily torn, as seen in one of the structures; one highly wrinkled coat is shown in this photograph. The raised ring effect is contrasted with a non-disrupted granule which has a significant shadow. The thickness of the membrane shell remaining after the removal of PHB was estimated to be about 150–200 Å thick. Further structural detail relating broken PHB granules, fragments, and loosened membranes may be observed in Pl. 3, fig. 12, and Pl. 4, fig. 13. A close examination of Pl. 3, fig. 11, to Pl. 4, fig. 13 shows material which looks somewhat different from intact granules and is probably extruded crystalline polymer. This material is quite like the 'lath'-shape crystals of PHB formed when a pure solution of PHB in chloroform was treated with ethanol, as shown in Pl. 4, fig. 14. Properties of these crystals have been described elsewhere (Alper *et al.* 1963).

DISCUSSION

The application of the carbon-replica technique to the investigation of PHB granules from *Bacillus* organisms has made possible the identification of discrete membrane-like structures which encase the granules. This technique of Bradley & Williams (1957) has recently been used by Hopwood & Glauert (1961) to increase the amount of structure observable on the surface of bacteria. Details of the structure of a fibrous coat around spores were detected in *Streptomyces violaceoruber*. Earlier studies of PHB inclusions, using Sudan Black B staining, phase microscopy, and electron microscopy of both gross and ultra-thin sections, have failed to establish clearly the presence of such a membrane. The first two methods could not detect such a structure because the light microscope could not resolve it. Bacteria containing PHB inclusions have been studied with the electron microscope; Chapman (1956) studied *Bacillus cereus*, and Cohen-Bazire & Kunisawa (1963) studied *Rhodospirillum rubrum*. Chapman's micrographs revealed discrete inclusions and instances of coalesced PHB granules; no membranous covering of the inclusions was discussed in that paper. Cohen-Bazire & Kunisawa characterized the reserve material of *R. rubrum* produced under different cultural conditions and reported the absence of a limiting membrane for glycogen and PHB granules. The failure to identify a membrane around PHB granules was probably due to the thinness of such a structure and the method of treatment. The PHB was soluble in the embedding material (Vestopal) which would have destroyed the granules' membrane. Thin sections of bacteria only showed empty areas where the PHB granules were initially present. Electron micrographs of shadowed lipid inclusions (PHB) from *B. megaterium* KM and *B. cereus* were reported by Weibull (1953) and Williamson & Wilkinson (1958), respectively.

Membrane systems have been identified in bacteria in several instances. Outstanding is the example of pigment-bearing particles (chromatophores) in *Rhodospirillum rubrum* which are surrounded by a membrane (Vatter & Wolfe, 1958). Other examples of membranous organelles were cited by Murray (1960) who reviewed the internal structure of the bacterium. Our evidence suggests that the PHB granules are membranous organelles but their nature and complexity needs further investigation. The most likely function for PHB is as a storage material (Macrae & Wilkinson, 1958; Doudoroff & Stanier, 1959; Schlegel *et al.* 1961). How the membrane regulates the metabolism of this storage product is an interesting question; probably even more exciting is the suggestion that perhaps the metabolism of other types of storage materials (i.e. starch, glycogen, polyphosphates) may be regulated by a membranous system. Answers to such questions should prove to be of fundamental importance in understanding the over-all metabolism of bacterial storage substances.

This work was supported by the Atomic Energy Commission, U.S.A., under contract no. AT 30-1(2038) issued to D. G. Lundgren, and by a grant from the National Institutes of Health, U.S.A., issued to J. M. Merrick. The suggestions and encouragement of Drs R. Alper and R. Marchessault are warmly acknowledged, for without them much of this work would not have been possible.

REFERENCES

- ALPER, R., MARCHESSAULT, R. & LUNDGREN, D. (1962). Characterization of poly-beta-hydroxybutyrate from *Bacillus cereus*. *Abs. Eighth Int. Congr. Microbiol., Montreal*, p. 19.
- ALPER, R. E., LUNDGREN, D. G. & MARCHESSAULT, R. H. (1963). The characterization of poly-beta-hydroxybutyrate from *Bacillus cereus* and other bacterial species. *Bact. Proc.* p. 49.
- ALPER, R. E., LUNDGREN, D. G., MARCHESSAULT, R. H. & COTE, W. A. (1963). Some physical properties of poly- β -hydroxybutyrate (PHB). Polymer Division, Preprints, 145th National Meeting, Sept. 1963.
- BRADLEY, D. E. & WILLIAMS, D. J. (1957). An electron microscope study of the spores of some species of the genus *Bacillus* using carbon replicas. *J. gen. Microbiol.* **17**, 75.
- CHAPMAN, G. B. (1956). Electron-microscopy of ultra-thin sections of bacteria. II. Sporulation of *Bacillus megaterium* and *Bacillus cereus*. *J. Bact.* **71**, 348.
- COHEN-BAZIRE, G. & KUNISAWA, R. (1963). The fine structure of *Rhodospirillum rubrum*. *J. Cell Biol.* **16**, 401.
- DOUDOROFF, M. & STANIER, R. (1959). The role of poly- β -hydroxybutyric acid in the assimilation of organic carbon by bacteria. *Nature, Lond.* **18**, 1440.
- HESTER, A. S. (ed.) (1963). Bacteria produce polyester thermoplastic. Solvents extract high molecular weight isotactic poly- β -hydroxybutyric acid from bacterial cells. *Chem. Engng News*, 18 March, p. 40.
- HOPWOOD, D. A. & GLAUERT, A. M. (1961). Electron microscope observations on the surface structures of *Streptomyces violaceoruber*. *J. gen. Microbiol.* **26**, 325.
- LUNDGREN, D. G. & BOTT, K. F. (1963). Growth and sporulation characteristics of an organic sulfur-requiring auxotroph of *Bacillus cereus*. *J. Bact.* **86**, 462.
- MACRAE, R. M. & WILKINSON, J. F. (1958). Poly- β -hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *J. gen. Microbiol.* **19**, 210.
- MERRICK, J. M. & DOUDOROFF, M. (1961). Enzymatic synthesis of poly- β -hydroxybutyric acid in bacteria. *Nature, Lond.* **189**, 890.
- MERRICK, J. M., DELAFIELD, F. P. & DOUDOROFF, M. (1962). Hydrolysis of poly- β -hydroxybutyrate by intracellular and extracellular enzymes. *Fed. Proc.* **21**, 228.
- MURRAY, R. G. E. (1960). The internal structure of the cell. In *The Bacteria*. Ed. by I. C. Gunsalus & R. G. Stanier. Vol. 1, p. 35. New York: Academic Press.
- SCHLEGEL, H. G. & GOTTSCHALK, G. (1962). Poly- β -hydroxy-buttersäure, ihre Verbreitung, Funktion und Biochemie. *Angew. Chem.* **74**, 342.
- SCHLEGEL, H. G., GOTTSCHALK, G. & VON BARTHA, R. (1961). Formation and utilization of poly- β -hydroxybutyric acid by Knallgas bacteria (*Hydrogenomonas*). *Nature, Lond.* **191**, 463.
- SIERRA, G. & GIBBONS, N. E. (1962). Production of poly- β -hydroxybutyric acid granules in *Micrococcus halodenitrificans*. *Canad. J. Microbiol.* **8**, 250.
- SLEPECKY, R. A. & LAW, J. H. (1960). A rapid spectrometric assay of α , β -unsaturated acids and β -hydroxybutyric acid. *Analyt. Chem.* **32**, 1697.
- VATTER, A. E. & WOLFE, R. S. (1958). The structure of photosynthetic bacteria. *J. Bact.* **75**, 480.
- WEIBULL, C. (1953). Characterization of the protoplasmic constituents of *Bacillus megaterium*. *J. Bact.* **66**, 696.
- WILLIAMSON, D. H. & WILKINSON, J. F. (1958). The isolation and estimation of the poly- β -hydroxybutyrate inclusions of *Bacillus* species. *J. gen. Microbiol.* **19**, 198.

EXPLANATION OF PLATES

(The scale marks represent 0.5μ)

PLATE 1

Figs. 1–4. Electron micrographs of both carbon replicas of PHB isolated from *Bacillus cereus* and *B. megaterium* and *B. cereus* spores shadowed with germanium.

Fig. 1. Membrane fragments are shown associated with the granules. The surfaces of the granules are varied in smoothness and suggest a laminated structure. $\times 40,000$.

Fig. 2. The rod-like granules show a varied surface as well as evidence of a membrane covering. $\times 25,000$.

Fig. 3. *B. cereus* ATCC 4342 spores showing sculptured surface and a typical loose exosporium. $\times 33,000$.

Fig. 4. Carbon replica of germanium shadowed 'native' granules from *B. megaterium* showing a defined core and outer membranous layer. $\times 40,000$.

PLATE 2

Figs. 5–8. Electron micrographs of both carbon replica and germanium shadowed 'native' PHB granules isolated from *Bacillus megaterium*.

Fig. 5. Carbon replica of germanium shadowed 'native' granules showing varied surface structure. Torn membrane fragments are shown around a number of these bodies. $\times 35,000$.

Fig. 6. Carbon replica of 'native' PHB granules showing an entire surface membrane. The large granule shows a definite wrinkle in the membrane covering. $\times 60,000$.

Fig. 7. 'Native' PHB granules germanium shadowed showing a dense core with a well-defined, less dense outer layer. $\times 28,000$.

Fig. 8. Granules with smooth surfaces showing broken membranes and a more dense peripheral structure which may be multi-layered. The peripheral dense appearance is due to its being 'edge-on' in the preparation. $\times 50,000$.

PLATE 3

Figs. 9–12. Electron micrographs of carbon replicas of enzymically active 'native' PHB granules both untreated and treated with sodium lauryl sulphate.

Fig. 9. 'Native' granules in association with a vegetative cell. $\times 38,000$.

Fig. 10. Intact 'native' granules which are the type readily susceptible to hydrolysis by cell-free extracts of *Rhodospirillum rubrum*. $\times 40,000$.

Fig. 11. A collection of membrane coats and a single PHB granule showing a rough surface. The coats resemble craters. $\times 34,000$.

Fig. 12. Granules showing fragmented membranes and a coat. $\times 34,000$.

PLATE 4

Figs. 13, 14. Electron micrographs showing PHB granules treated with sodium lauryl sulphate and isolated crystals of PHB shadowed with germanium.

Fig. 13. Membrane fragments and coats and suspected crystalline PHB material. $\times 30,000$.

Fig. 14. Single crystals of PHB grown in the test tube. $\times 30,000$.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Fig. 14

D. G. LUNDGREN, R. M. PFISTER AND J. M. MERRICK