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Characterization of Poly-β-Hydroxybutyrate Extracted from Different Bacteria

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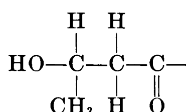
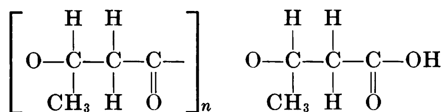
ABSTRACT

LUNDGREN, D. G. (Syracuse University, Syracuse, N.Y.), R. ALPER, C. SCHNAITMAN AND R. H. MARCHESSAULT. Characterization of poly-β-hydroxybutyrate extracted from different bacteria. J. Bacteriol. **89**:245–251. 1965.—Poly-β-hydroxybutyrate (PHB) from different bacterial genera was studied with regard to its crystal structure, infrared absorption, intrinsic viscosity, and electron microscopy. All PHB samples precipitated from dilute chloroform solution gave identical X-ray diffractograms confirming uniformity of crystal structure, and uniformity of molecular structure, based on the similarity of the recorded infrared spectra, was also established. Crystal morphology was also similar, showing the reported "lath" shape structure for purified polymer from *Bacillus cereus*. Intrinsic viscosity ranged from 0.04 to 11.5 depending upon the polymer treatment; polymer molecular weights, based upon viscometry, could be estimated to range from 1,000 to 250,000. It is concluded that the same basic molecule is involved in all PHB present in the bacterial kindgom.

The general physical properties of poly-β-hydroxybutyrate (PHB) extracted from *Bacillus cereus* and *Rhizobium* species have recently been reported (Alper et al., 1963), as well as an examination of the ultrastructure of "native" granules after various chemical and physical treatments (Lundgren, Pfister, and Merrick, 1964).

There is good evidence that the structure for the polymer is:





and that the molecular weight can vary depending upon bacterial growth conditions, growth stage, and the chemical extraction procedures. Further, the polymer was found to be crystalline in vivo, and exhibits a consistent morphological state as viewed in the electron microscope after precipitation from a dilute chloroform solution by addition of ethanol. From X-ray data, a fiber repeat of 5.9 A was found, a value which can only be reconciled with some form of helical conformation in the solid state (Alper et al., 1963).

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Previous studies were confined to PHB taken from only two organisms. To determine if there is a common structural and physical basis for PHB, we collected bacterial PHB from many different investigators representing polymer from 11 different genera, and analyzed these with regard to their molecular weight, infrared-absorption spectrum, X-ray diffraction pattern, and precipitate structure (determined by use of the electron microscope). Results of these comparative studies are reported in this paper.

MATERIALS AND METHODS

The sources of the different polymer samples are shown in Table 1.

X-ray diffractograms of PHB were made with thin pieces of extracted film or recrystallized powder placed in a sample holder and supported in front of the X-ray beam. A Norelco diffraction unit (North American Philips Co., Mount Vernon, N.Y.) with a copper target X-ray tube operated at 35 kv and 20 ma was the X-ray source. A nickel filter served to isolate CuKα radiation which was used to record the diffraction patterns on Kodak "no screen" X-ray film in a flat plate camera. Exposure times ranged from 1.5 to 15 hr, and film to-sample distance was 4 cm.

For infrared-absorption analysis, enough poly-

TABLE 1. *Sources of the different polymer samples*

Organism	Sample	Source
Hydrogenomonas No. 16 *Chromatium okenii* *Azotobacter chrococcum* *Micrococcus denitrificans*	Polymer extracted from hypochlorite-dissolved cells, and the residue washed and dissolved in hot chloroform and dried.	H. G. Schlegel, Institute of Microbiology, University of Gottingen, Germany
Azotobacter chrococcum	Ether precipitated from a chloroform solution.	H. G. Schlegel
Spirillum normaal	Polymer extracted from hypochlorite-dissolved cells, and the residue washed and dissolved in hot chloroform, and reprecipitated with ether.	J. H. Becking, Laboratorium voor Microbiologie, Wageningen/RUC, The Netherlands
Bacillus megaterium KM *Rhodospirillum rubrum*	Polymer extracted from hypochlorite-dissolved cells, and extracted with acetone and ether.	M. Doudoroff, Department of Bacteriology, University of California, Berkeley
Pseudomonas saccharophila *Rhodospirillum rubrum* *Bacillus megaterium* KM	Polymer from cells not treated with hypochlorite, but extracted with acetone and ether and precipitated from chloroform.	M. Doudoroff
Spirillum itersonii *Spirillum serpens*	Polymer extracted from hypochlorite-dissolved cells with chloroform.	J. F. Wilkinson, Department of Bacteriology, University of Edinburgh, Scotland
Rhizobium (unidentified species)	Acetone-dried cells, extracted with hot chloroform.	F. X. Werber, Grace Chemical Co., Clarksville, Md.
Micrococcus halodenitrificans	Ballotini bead-disrupted cells were dried and extracted with chloroform and dioxane in a Soxhlet apparatus. The extract was evaporated to dryness, the residue was redissolved in hot chloroform, and the polymer was precipitated with acetone-ether.	G. Sierra, Department of Biochemistry, Ontario Research Foundation, Toronto, Ontario, Canada
Ferrobacillus ferrooxidans	Washed dried cells were extracted with chloroform-methanol in a Soxhlet apparatus, and the extracted material was dried to a film.	R. Mahoney, Department of Bacteriology and Botany, Syracuse University, Syracuse, N.Y.
*Lampropedia hyalina**	Washed dried cells were extracted with hot chloroform, and the polymer was dried to a film.	H. W. Seeley, Department of Bacteriology, Cornell University, Ithaca, N.Y.

* Only an X-ray analysis was made on this PHB sample. The supplied PHB could not be redissolved in chloroform. It has been our experience that when PHB films are obtained from chloroform evaporation where heat is applied, a change in structural configuration must occur, for the PHB is no longer soluble in chloroform.

mer solution (1 to 2 mg in chloroform) was placed in a small beaker and evaporated to dryness under a stream of filtered air. The residue was dissolved in a few drops of chloroform and smeared on a silver chloride plate (5 × 10 mm) and air dried. The plate was mounted in a double beam Baird-Atomic Infrared Spectrophotometer (Baird-Atomic Inc., Cambridge, Mass.), and the reference beam was adjusted to give 95 to 105% transmittance at 13.0 μ. A scan was made from 2 to 16 μ at a speed of about 1.0 μ/min, and with a programmed slit opening of 2×. Some double thickness samples were also prepared by coating both sides of the silver chloride plate and making the scan over 2 to

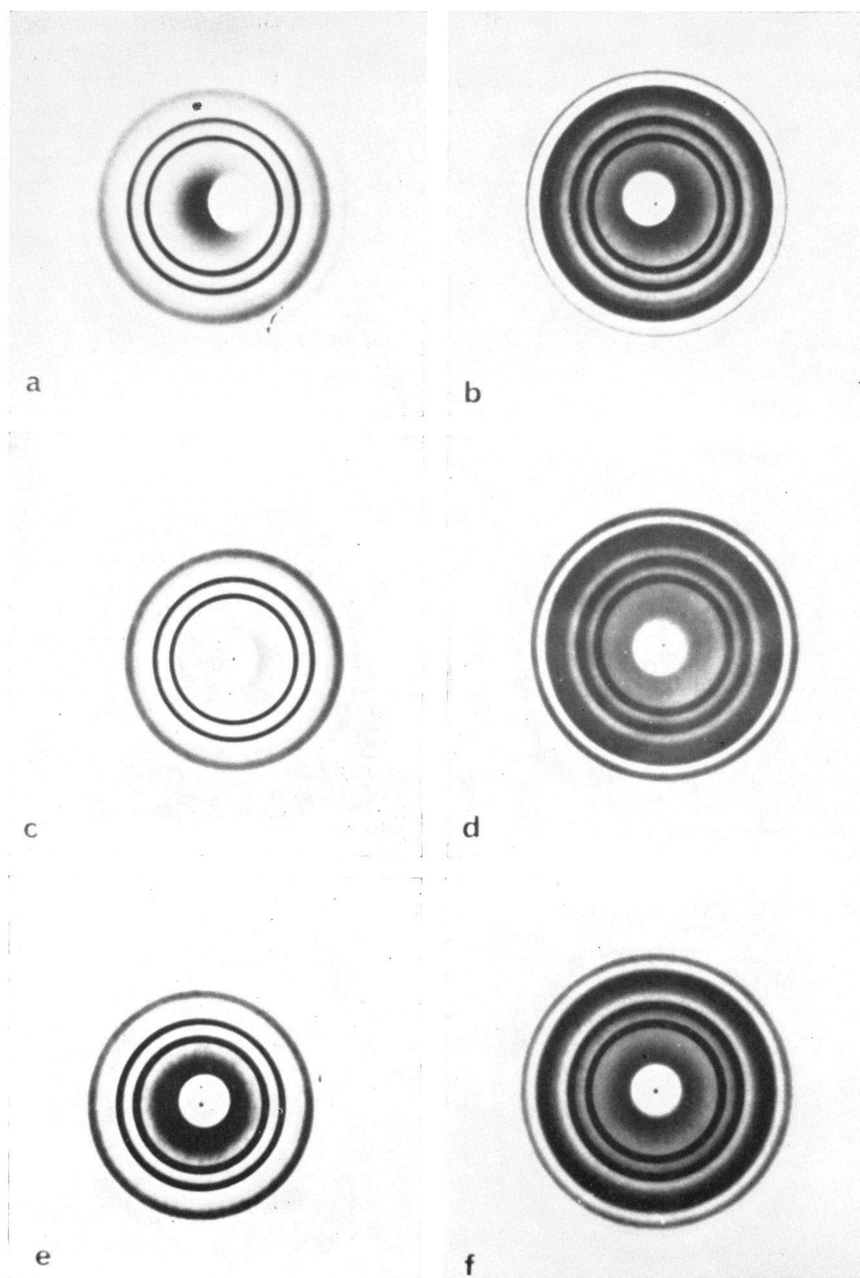

FIG. 1. *X-ray diffractograms of PHB isolated from different bacteria. (a) Hydrogenomonas No. 16, (b) Ferrobacillus ferrooxidans, (c) Azotobacter chrococcum, (d) Micrococcus halodenitrificans, (e) Rhodospirillum rubrum, (f) Bacillus megaterium KM.*

8 μ at a speed of 0.5 μ/min after adjusting the transmittance to 95% at 5.0 μ.

Intrinsic viscosity measurements were made at 30 C, with chloroform as a solvent, in a Ubbelohde viscometer with a solvent-flow time of 129.2 sec. All chloroform solutions of polymer were first clarified by filtration through a coarse sintered-glass filter, and any filtrate having less than a 10-ml volume was brought to this mark by adding more chloroform. A 7-ml amount of solution was transferred to the viscometer, and the remaining 3.0 ml was used for a dry-weight determination. The final concentration of the solution in the viscometer ranged from 0.2 to 1.5 g/100 ml.

TABLE 2. *Intrinsic viscosity and approximate molecular weight of poly-β-hydroxybutyrate from various sources*

Organism	Isolation treatment	Intrinsic viscosity	Approximate molecular wt
Hydrogenomonas No. 16	Hypochlorite	0.09	2,000
Micrococcus denitrificans	Hypochlorite	1.05	22,000
Chromatium okenii	Hypochlorite	0.50*	11,000
Azotobacter chrococcum (recrystallized)	Hypochlorite	0.10*	2,000
A. chrococcum	Hypochlorite	0.23	5,000
Spirillum normaal	Hypochlorite	0.09	2,000
Bacillus megaterium KM	Hypochlorite	0.37	8,000
B. megaterium KM	No hypochlorite	0.06*	1,000
Rhodospirillum rubrum	Hypochlorite	0.09*	2,000
R. rubrum	No hypochlorite	0.20*	5,000
Pseudomonas saccharophila	No hypochlorite	11.45	256,000
S. itersonii	Hypochlorite	0.04*	1,000
S. serpens	Hypochlorite	0.05*	1,000
M. halodenitrificans	Hypochlorite	0.05*	1,000
Ferrobacillus ferrooxidans	No hypochlorite	2.60	59,000
Rhizobium sp.	No hypochlorite	5.60	128,000

* Approximate values.

By definition, intrinsic viscosity, $[\eta]$, is

$$\lim_{c \to o} \eta_{sp}/c = \frac{1}{c}\left(\frac{t_s - t_o}{t_o}\right)$$

where η_{sp} = specific viscosity, t_s = solution flow time in seconds, t_0 = solvent flow time in seconds, and c = concentration in grams per 100 ml. The reduced specific viscosity, η_{sp}/c, was plotted against concentration for all samples, and for at least three concentrations. Extrapolation to $c = o$ yielded the intrinsic viscosity which is related to molecular weight. Results were inaccurate when sample size was limited; in these cases the intrinsic viscosity is assumed to be equal to η_{sp}/c, and the approximate value is reported in Table 2.

Electron micrographs of polymer crystals were recorded for samples precipitated from a 0.1% chloroform solution by addition of 95% ethanol (Alper et al., 1963). The suspension was evaporated onto copper grids, shadowed with chromium, and examined in a RCA-2D electron microscope at a magnification of 7,000. Pictures were recorded on Kodak (2 × 10) lantern slide plates and enlarged photographically.

RESULTS AND DISCUSSION

X-ray diffractograms of PHB from all isolates were similar to the one recorded for *B. cereus* (Alper et al., 1963). The typical X-ray powder diagrams for six different samples are seen in Fig. 1; from the relative intensities and diameter ratios for the various diffraction rings, it was established that these were identical patterns to those recorded for *B. cereus* polymer. The importance of these data in establishing that a regular helical conformation of the molecule exists in the crystal has already been mentioned (Alper et al., 1963). Therefore, it appears that bacteria in general synthesize the same polymer, based on the monomer (β-hydroxybutyric acid), which then gives rise to this unique crystalline structure upon crystallization.

All PHB samples yielded essentially identical infrared spectra with the major absorption peak at 5.7 μ. The latter corresponds to the ester carbonyl stretching mode. The low viscosity samples (*Bacillus megaterium*) showed distinct −OH-stretching absorption peaks at 2.9 μ which corresponded to polymer end groups. Prolonged heating of the samples at 100 C in air caused the appearance of a new band at 6.25 μ corresponding to C=C absorption. The latter almost certainly arises from a simultaneous depolymerization and end dehydration of the sample. The infrared spectra of PHB from three organisms (Fig. 2) all compared well with the

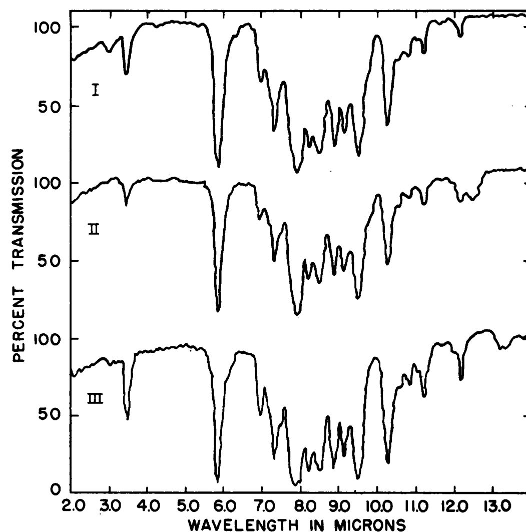

FIG. 2. *Infrared-absorption spectra of PHB isolated from different bacteria. (I) Bacillus megaterium, (II) Pseudomonas saccharophila, (III) Ferrobacillus ferrooxidans.*

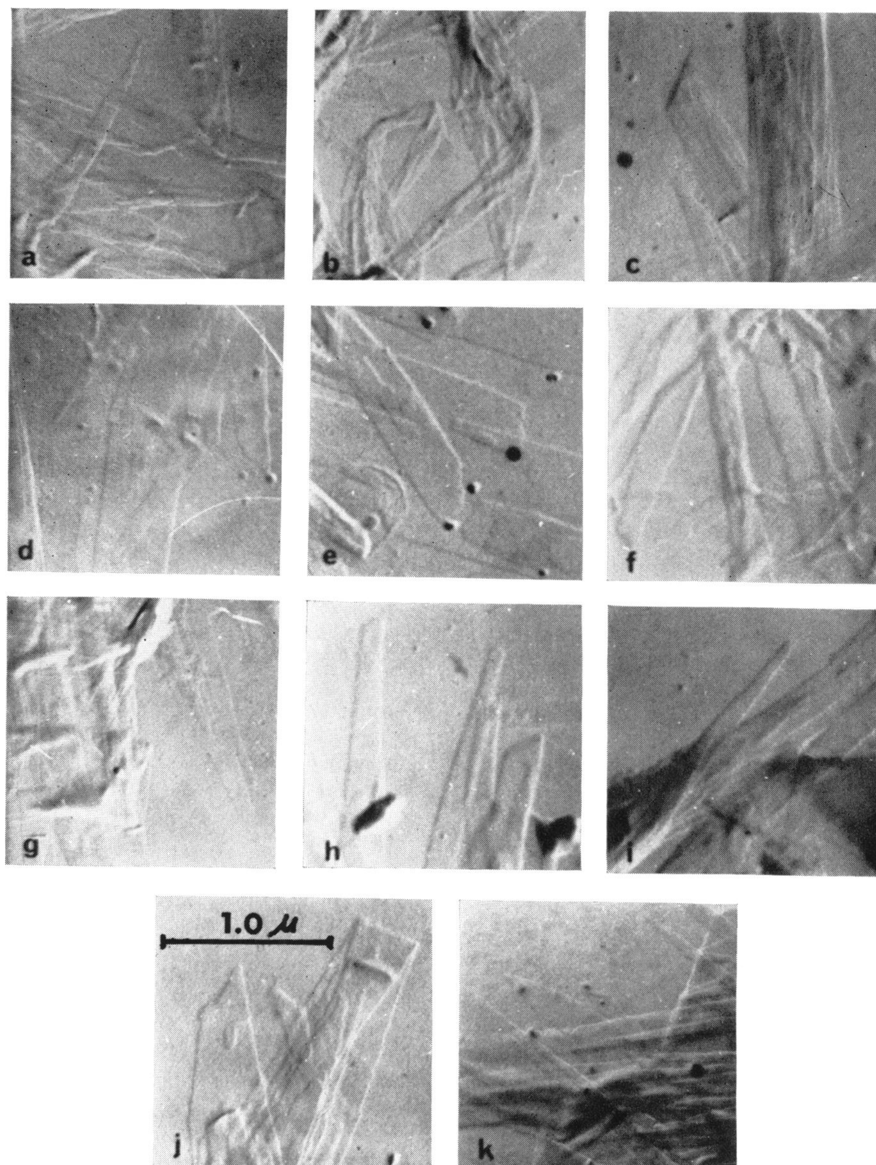

FIG. 3. *Electron microscopy of precipitated crystals of PHB isolated from different bacteria. (a) Azotobacter chrococcum, (b) Micrococcus denitrificans, (c) Spirillum normaal, (d) Rhodospirillum rubrum, (e) Chromatium okenii, (f) Pseudomonas saccharophila, (g) Micrococcus halodenitrificans, (h) Spirillum serpens, (i) Azotobacter sp., (j) Hydrogenomonas No. 16, (k) Bacillus megaterium KM. The scale for all figures is shown in section (j) and is equal to 1.0 μ.*

spectra of PHB reported for *B. megaterium* and *Sphaerotilus natans* by Rouf and Stokes (1962). A few bands in the region of 12 to 13 μ of our spectra appear to be due to contamination, as they are not present in recrystallized preparations.

There was a wide spread of values (11.45 to 0.04) in the intrinsic viscosity of the various PHB samples (Table 2). It is possible to convert these data into approximate polymer molecular weights, realizing that we may be in error by a factor of two. Alper et al. (1963) reported the molecular weight of *Rhizobium* PHB (solvent extraction) to be 128,000 as determined by

osmometry; the intrinsic viscosity of this sample was 5.6. Assuming molecular weight is directly proportional to viscosity, the 11.45 value would correspond to a molecular weight of 256,000, and the lowest molecular weight would be about 1,000.

The viscosity data shows that samples prepared via the alkaline hypochlorite method have uniformly low molecular size. Experience has shown (Baptist, 1962) that direct solvent extraction of the dried cells is much less degradative, although traces of water or alcohol can cause depolymerization during extraction due to ester interchange. The sample of highest molecular weight was polymer from *Pseudomonas saccharophila*. This sample was not treated with hypochlorite. Why other polymer samples not treated with hypochlorite had lower molecular weights cannot be definitely answered, but we know that polymer structure is complex and subject to breakdown from physical and chemical agents (Lundgren et al., 1964; Merrick, Lundgren, and Pfister, 1964). These facts were not known at the time the samples were prepared. Polymer synthesis and breakdown is undoubtedly of an equilibrium nature (Merrick and Doudoroff, 1964), and isolation of undegraded polymer is an unrealistic goal.

Although we do not report the melting points for these samples, other workers have reported a wide range of melting points for PHB isolated from different bacterial species (Schlegel and Gottschalk, 1962). The lowest melting points reported for PHB are those of Lemoigne (1927, 1946) with values of 114, 120, and 157 C. The highest value reported is 188 C for *Chromatium* PHB (Schlegel and Gottschalk, 1962). Generally, melting point values range from about 168 to 173 C. The different melting point values are related to polymer fractions which have undergone different degrees of degradation yielding a polyester with a relatively large fraction of chain ends (i.e., low degree of polymerization, DP). It is well established in polymer chemistry that an end group acts as an impurity and lowers polymer melting points (Mandelkern, 1956). End groups of polymer chains of infinite lengths seldom occur in the crystallites.

Micrographs of crystals of PHB from different bacteria revealed crystals of similar morphologies (Fig. 3). PHB samples with low viscosities were very slow to recrystallize and yielded few crystals, which probably is a reflection of the low DP, and the resulting polymer was more soluble in the alcohol. Generally, the "lath"-shape crystals were evident with folds seen in the laths. In some instances, large crystals showed some lamellar morphology, but not as extensive as that noted for *B. cereus* PHB crystals (Alper et al., 1963). This is probably a procedural limitation, for no great care was taken in growing large crystals from polymer solutions.

Results of this study support the conclusion that the same basic molecule is found in all bacteria, and that the polyester is of importance to the microbial world. The polymeric character of the material is to be emphasized, because the first physically determined molecular weight (Williamson and Wilkinson, 1958) was about 5,000. The sensitivity of the material to acid and alkaline conditions cannot be overemphasized, and this fact is one of the principal reasons why many of the samples of PHB isolated so far have been of extremely low molecular weight. This fact was clearly evident from the work of Péaud Lenoël and Kepes (1952) who were the first to develop a direct extraction method with chloroform which yielded a sample of relatively high intrinsic viscosity.

The role of PHB in endogenous metabolism has been reviewed by Dawes and Ribbons (1964), and this seems to be one of the major roles played by the PHB storage product. It is not known why it exists in a crystalline state within bacteria, but we hope that studies such as the one reported will serve as part of the foundation upon which to build information leading to a full understanding of the relationship to structure and function.

ACKNOWLEDGMENTS

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