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Kenneth Nickerson
UNL, knickerson1@unl.edu

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Purification of Poly-β-Hydroxybutyrate by Density Gradient Centrifugation in Sodium Bromide

KENNETH W. NICKERSON
School of Life Sciences, University of Nebraska, Lincoln, Nebraska 68588

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Fractionation of fully sporulated cultures of *Bacillus thuringiensis* by density gradient centrifugation in NaBr produced two bands which were identified as poly-β-hydroxybutyrate. This technique generated high yields of membrane-bound and unbound granules of exceptional purity and degree of polymerization.

Poly-β-hydroxybutyrate (PHB) is the simplest known biologically important polymer (2, 3). It is commonly found in the soil and is produced by a wide variety of microorganisms. PHB constitutes an ideal storage compound, since it is highly reduced, virtually insoluble, and exerts negligible osmotic pressure. Traditional interest in PHB has centered on its role in bacterial sporulation (7), but more recently it has also been viewed as a potentially useful plastic (5). Significantly, the difficulty of separating the PHB from the cell debris has so far been the primary technical obstacle to its commercial exploitation. The ability to hydrolyze PHB is a useful taxonomic criterion for distinguishing among members of the genus *Pseudomonas* (13), limited solely by the difficulty of preparing PHB suspensions (12). The present communication reports the ready purification of two PHB fractions from sporulated cultures of *Bacillus thuringiensis* by density gradient centrifugation in NaBr.

An overnight culture (50 ml) of *B. thuringiensis* subsp. HD-1 was inoculated into 5 liters of a glucose-yeast extract-salts medium (10) in a 7.5-liter fermentor jar. The culture (initial pH of 7) was grown with aeration (4,000 ml/min) and agitation (180 rpm) for 5 days at 22°C in a New Brunswick Scientific Co. FS-307 fermentor. After sporulation, the cells were allowed to autolyze, whereupon the culture was harvested and fractionated on NaBr density gradients (1). The samples were treated in a Waring blender for 10 min before centrifugation. No additional steps to liberate the PHB or to remove non-PHB lipids were necessary. Figure 1 illustrates the presence of two peaks at densities considerably lower than those characteristic of the spores and of entomocidal protein crystals also produced by *B. thuringiensis*. Yields from 5 liters of culture were ca. 50 mg for PHB fraction 1 and 200 mg for PHB fraction 2. The centrifugation conditions were as described previously (1) except that the gradient shape was modified to optimize PHB separation, and, consequently, the spores were pelleted against the rotor wall. This separation was repeated five times with virtually identical results. Repurification of the two presumptive PHB bands gave peaks of unchanged shape and position. The particles contained in these peaks were soluble in $CHCl_3$, exhibited a melting point of 182 to 185°C, and when viewed by scanning electron microscopy (11), appeared as a reasonably homogeneous group of spherical-to-ovoid granules of ca. 0.35-μm diameter. The heavier fraction (PHB fraction 2) did not contain any detectable protein (11) or carbohydrate (6). Coupled with the observed densities, these properties are consistent with those expected of PHB granules (4, 14). This identification was confirmed by both elemental analysis (Table 1) and the accepted test for PHB described by Law and Slepecky (8). In the latter procedure, the polymer is hydrolyzed to β-hydroxybutyrate, which undergoes dehydration to crotonic acid in concentrated sulfuric acid. The absorbance of the double bond-containing crotonic acid is measured at 235 nm. With sodium β-hydroxybutyrate as a standard, lyophilized samples from both peaks were found to be 100 ± 5% PHB.

The two PHB fractions obtained (Fig. 1) appear to be of exceptional purity. Their elemental analyses (Table 1) were closer to the theoretical values than those of previously reported samples prepared by hypochlorite treatment and chloroform extraction (8, 14). Additionally, their high melting points (182 to 185°C) indicate a very high degree of polymerization (2). However, the existence of two separable PHB fractions raises the question of how they differ. One likely explanation, consistent with the elemental analyses (Table 1), is that the less-dense fraction is still bound by the thin membrane known to surround PHB granules in vivo (2, 9). For instance, Lundgren et al. (9) used electron microscopy of thin sections to demonstrate the existence of a membrane 6 to 8 nm thick around the PHB granules in *Bacillus cereus*. Griebel et al.

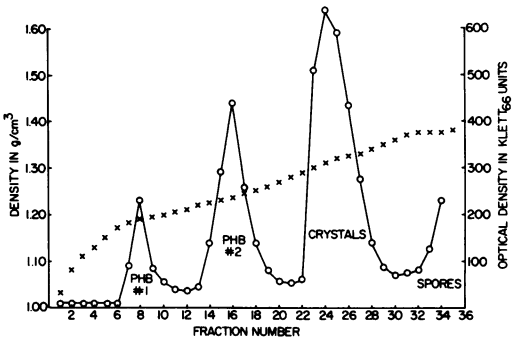

FIG. 1. Separation of two PHB bands by density gradient centrifugation in NaBr. ×, Density in grams per cubic centimeter; ○, $Klett_{66}$ units.

TABLE 1. Elemental analysis of the PHB fractions from *B. thuringiensis*

Element	PHB (%)[a]		
	Fraction 1	Fraction 2	Theoretical
Carbon	55.67	55.77	55.80
Hydrogen	7.06	7.08	7.03
Nitrogen	0.32	—	0
Oxygen	35.09	37.02	37.17
Phosphorous	0.007	—	0
Sulfur	ND	—	0

[a] PHB fraction 1 is the lighter band at ca. 1.19 g/cm³, and PHB fraction 2 is the denser band at ca. 1.23 g/cm³ (Fig. 1). The theoretical values are calculated for $(C_4H_6O_2)_n$. ND, Not done; —, Not detected. All analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

(4) determined that native PHB granules from *Bacillus megaterium* contained 2% protein and 0.5% non-PHB lipid. Support for the idea that the less-dense PHB fraction is composed of native membrane-bound granules is derived from the fact that they alone could be stained with Sudan black. PHB granules in fixed bacterial preparations are intensely stained by Sudan black, but purified polymer and all previously isolated granules (2) do not take up the dye, presumably because their surrounding membranes have been removed during purification.

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