VOLUME 116, NUMBER 7
APRIL 6, 1994

JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY

Amorphous, Biomimetic Granules of Polyhydroxybutyrate: Preparation, Characterization, and Biological Implications†

Daniel M. Horowitz and Jeremy K. M. Sanders*

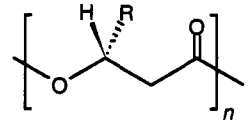
Contribution from the University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Received November 12, 1993®

Abstract: It is a striking feature of the bacterial storage polymer poly-3-hydroxybutyrate (PHB) that, although the isolated polymer is highly crystalline, native PHB storage granules in cells are only found in an amorphous, mobile state. It has recently been proposed that the failure of PHB granules to crystallize is simply the result of slow nucleation kinetics that are operative for small particles. In support of this new model, we report here a straightforward procedure by which pure crystalline PHB can be reconstituted into submicron-size artificial granules. In the artificial granules, a synthetic surfactant has been substituted for the native granule coating. The artificial granules are amorphous and stable in suspension, and they are essentially indistinguishable from their native counterparts in terms of size, morphology, molecular mobility, and density. Furthermore, when the surfactant coating is removed from the artificial granules by dialysis, the granules crystallize, verifying the nucleation hypothesis. *In vivo*, the PHB granule surface is likely to consist of both protein and phospholipid; *in vitro* it is possible to prepare amorphous PHB granules which are stabilized solely by phospholipid. PHB artificial granule latexes crystallize well on drying and annealing, making them potentially useful in the preparation of polymer coatings. Artificial amorphous granules may also be prepared from other bacterial polyhydroxyalkanoates (PHAs) and from blends of incompatible polyesters.

Introduction

Poly-3-hydroxyalkanoates (PHAs, **1**) are linear, hydrophobic polyesters that occur naturally in a wide variety of bacteria and other organisms.[1] In bacteria the polymers function as energy and carbon storage materials. Poly-R-3-hydroxybutyrate (PHB, **2**) was the first of the PHAs to be discovered and is the most widespread in nature. PHAs have attracted substantial industrial interest as biologically derived, fully biodegradable thermoplastics; poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV or Biopol, **3**) is now produced commercially by fermentation for use in packaging and other applications. Due to its absolute stereoregularity, isolated bacterial PHB crystallizes readily from the melt (T_m 180 °C) and achieves a high degree of crystallinity, typically about 70%.



1: PHA, R = various alkyl, alkenyl, and aryl groups (C_{1-11})
2: PHB, R = CH_3
3: PHBV (Biopol), R = CH_3, CH_2CH_3

As PHB is insoluble in water, the polymer *in vivo* occurs in the form of discrete inclusion bodies or "granules", which under

* Author for correspondence.

† A preliminary account of this work has appeared: Horowitz, D. M.; Clauss, J.; Hunter, B. K.; Sanders, J. K. M. *Nature* **1993**, *363*, 23. Abbreviations used: PHB (poly-3-hydroxybutyrate), PHBV (poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate), PHO (poly-3-hydroxyoctanoate), PHA (poly-3-hydroxyalkanoate), CTAB (cetyltrimethylammonium bromide), SDS (sodium dodecyl sulfate), EDTA (ethylenediaminetetraacetate), BHT (butylated hydroxytoluene), WAXS (wide-angle X-ray scattering), O/W (oil-in-water).

® Abstract published in *Advance ACS Abstracts*, March 1, 1994.

(1) For reviews see: (a) Anderson, A. J.; Dawes, E. A. *Microbiol. Rev.* **1990**, *54*, 450–472. (b) Müller, H.-M.; Seebach, D. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 477–502.

0002-7863/94/1516-2695$04.50/0

appropriate cultural conditions may comprise up to 80% of the dry weight of the cell. The granules vary in size and number among different organisms, but in the commercial bacterium *Alcaligenes eutrophus*, a typical PHB-rich cell contains 8–12 granules, averaging 0.25 μm in diameter.[2] PHB is by far the major constituent of the granules; native granules from *Bacillus megaterium* were reported to contain 97.7% PHB together with 1.9% protein and 0.4% lipid,[3] the latter two presumed to form a surface coating around an essentially pure PHB core. The physical state of the PHB within the granules has been much debated.[4] PHB granules were characterized initially in the 1960s by carbon-replica electron microscopy[5] and X-ray diffraction.[6] It was concluded that PHB *in vivo* was crystalline with the same structure as the isolated polymer. This finding remained unchallenged until the late 1980s when studies of whole, PHB-producing cells by ^{13}C-NMR spectroscopy,[7] wide-angle X-ray scattering (WAXS),[8] and transmission electron microscopy[9] revealed unequivocally that the polymer *in vivo* is completely amorphous. ^{13}C-NMR spectroscopy proved to be an especially suitable technique for resolving this issue, as mobile polymer could be detected directly in intact, living bacterial cells in aqueous suspension. Crystalline PHB granules would display no NMR spectrum using conventional solution-state techniques; however, mobile amorphous polymer would be expected to exhibit sharp lines. It was found that the polymer *in vivo* does indeed have a readily observable spectrum at temperatures 50 °C above T_g but some 130 °C below T_m. As T_g for the homopolymer is 5 °C, the polymer in living cells at 30 °C is in an elastomeric state.

Isolation of the PHB granules by all but the mildest techniques has been found to lead to apparently irreversible crystallization. In early studies, biochemical "inactivation" of native granules, now understood to involve crystallization of the polymer, was associated with a variety of treatments, including excessive centrifugation, repeated freezing and thawing, and exposure to organic solvents, proteolytic enzymes, and heat.[10] In recent years, numerous hypotheses have been advanced to explain the metastable amorphous state of the polymer *in vivo*. The involvement of labile protein or lipid factors,[11] the existence of natural plasticizers,[12] keto–enol tautomerism,[13] and the existence of a water-stabilized extended chain conformation[14] have all been suggested. None of these hypotheses, however, have gained much direct experimental support.

Two groups proposed recently that the amorphous state of PHB *in vivo* can be explained by a simple physical–kinetic mechanism.[15] The essence of the kinetic model is that polymer that is newly synthesized in an amorphous mobile form within the granule will only crystallize as a result of spontaneous nucleation. It has long been recognized that the macroscopic rate of crystallization of a liquid polymer or other substance can be significantly retarded by subdividing the substance into a large number of small, physically isolated droplets.[16] In such systems, crystallinity in one droplet does not seed crystallization in other droplets. When crystallization does occur in a droplet, its propagation within that droplet is rapid. Bulk crystallization may be slowed dramatically provided that (1) the droplets are sufficiently small that only a small proportion contain nucleating contaminants and (2) the frequency of spontaneous nucleation within individidual droplets is low. Slow crystallization has been observed experimentally at temperatures well below T_m for isolated droplets of a variety of metals and small molecules[16,17] as well as for several polyolefins, polyethers, and polyamides.[18] In such systems, the fraction of droplets nucleated spontaneously with time (n/n_0) is given by a simple exponential function:[19]

$$n/n_0 = 1 - e^{-Iv t}$$

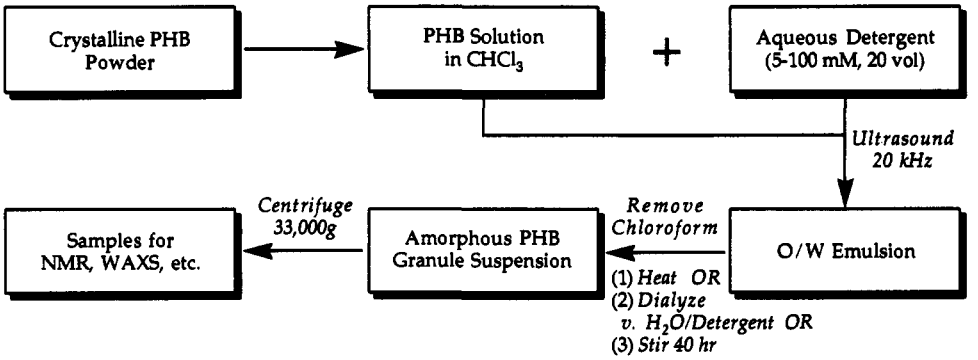
where I is the nucleation rate constant and v is the droplet volume.

As PHB occurs in quite pure form within native granules, which are extremely small and are isolated from their environs by a protein/lipid coat, it is proposed that the rate of PHB crystallization *in vivo* is governed by the rate of spontaneous nucleation events within individual granules. Since this rate depends upon the granule volume, and therefore on the third power of radius, its predicted value is surprisingly low for the submicron-size polymer storage granules. The upper limit for the rate constant I of spontaneous crystal nucleation in isolated PHB at 30 °C (the temperature of bacterial growth) is 2.5 events mm^{-3} s^{-1}.[20] The corresponding rate of nucleation, Iv, for a typical storage granule of diameter 0.25 μm is 2.0×10^{-11} events s^{-1}. In the absence of any perturbation that causes granule coalescence or exposure of the polymer to heterogeneous nucleation catalysts, an ensemble of native granules should thus exhibit a crystallization half-life of at least 3.4×10^{10} s, or >1000 year.

It is a consequence of this model for the physical state of PHB *in vivo* that it might be possible to recreate the amorphous state of the polymer *in vitro* using pure PHB.[15a] Specifically, the model predicts that the native state could be duplicated if it were possible to generate PHB that was (1) amorphous at the outset; (2) contained in discrete, physically separate microscopic particles; and (3) either relatively free of nucleating contaminants or else coated in such a way that direct exposure of the polymer to the potential nucleants would be minimized.[21] We show here that a remarkably straightforward procedure can be used to prepare artificial PHB granules that are amorphous and generally indistinguishable from their native counterparts. Once again, as in the case of the native granules contained in whole cells, NMR has been used in a simple and noninvasive manner to verify the

(2) Ballard, D. G. H.; Holmes, P. A.; Senior, P. J. *Recent Advances in Mechanistic and Synthetic Aspects of Polymerization*; Fontanille, M., Guyot, A., Eds.; D. Reidel Publishing Company: Dordrecht, 1987; pp 293–314.
(3) Griebel, R.; Smith, Z.; Merrick, J. M. *Biochemistry* **1968**, *7*, 3676–3681.
(4) Calvert, P. *Nature* **1992**, *360*, 535.
(5) Lundgren, D. L.; Pfister, R. M.; Merrick, J. M. *J. Gen. Microbiol.* **1964**, *34*, 441–446.
(6) Ellar, D.; Lundgren, D. G.; Okamura, K.; Marchessault, R. H. *J. Mol. Biol.* **1968**, *35*, 489–502.
(7) Barnard, G. N.; Sanders, J. K. M. *J. Biol. Chem.* **1989**, *264*, 3286–3291.
(8) (a) Kawaguchi, Y.; Doi, Y. *FEMS Microbiol. Lett.* **1990**, *70*, 151–156. (b) Amor, S. R.; Rayment, T.; Sanders, J. K. M. *Macromolecules* **1991**, *24*, 4583–4588.
(9) McIntosh, H. M. Ph.D. Thesis, University of York, England, 1988.
(10) (a) Merrick, J. M.; Doudoroff, M. *J. Bacteriol.* **1964**, *88*, 60–71. (b) Merrick, J. M.; Lundgren, D. G.; Pfister, R. M. *J. Bacteriol.* **1965**, *89*, 234–239.
(11) See ref 8a; cf. ref 3.
(12) Harrison, S. T. L.; Chase, H. A.; Amor, S. R.; Bonthrone, K. M.; Sanders, J. K. M. *Int. J. Biol. Macromol.* **1992**, *14*, 50–56.
(13) Barham, P. J.; Bennett, P.; Fawcett, T.; Hill, M. J.; Stejny, J.; Webb, J. Abstracts of the conference Biologically Engineered Polymers, Cambridge, U.K., July 31–August 2, 1989.
(14) (a) Lauzier, C.; Marchessault, R. H.; Smith, P.; Chanzy, H. *Polymer* **1992**, *33*, 823–827. (b) Lauzier, C.; Revol, J. F.; Marchessault, R. H. *FEMS Microbiol. Rev.* **1992**, *103*, 311–316.
(15) (a) de Koning, G. J. M.; Lemstra, P. J. *Polymer* **1992**, *33*, 3292–3294. (b) Bonthrone, K. M.; Clauss, J.; Horowitz, D. M.; Hunter, B. K.; Sanders, J. K. M. *FEMS Microbiol. Rev.* **1992**, *103*, 273–282.
(16) Vonnegut, B. *J. Colloid Sci.* **1948**, *3*, 563–569.
(17) (a) Mathias, S. F. *Trends Biotechnol.* **1991**, *9*, 370–372. (b) Turnbull, D. *J. Chem. Phys.* **1952**, *20*, 411–424. (c) Turnbull, D.; Cormia, R. L. *J. Chem. Phys.* **1961**, *34*, 820–831.
(18) (a) Cormia, R. L.; Price, F. P.; Turnbull, D. *J. Chem. Phys.* **1962**, *37*, 1333–1340. (b) Gornick, F.; Ross, G. S.; Frolen, L. J. *J. Polym. Sci., Part C* **1967**, *18*, 79–91. (c) Burns, J. R.; Turnbull, D. *J. Appl. Phys.* **1966**, *37*, 4021–4026. (d) Koutsky, J. A.; Walton, A. G.; Baer, E. *J. Appl. Phys.* **1967**, *38*, 1832–1839. (e) Burns, J. R.; Turnbull, D. *J. Polym. Sci. Part A2* **1968**, *6*, 775–782. (f) Barham, P. J.; Jarvis, D. A.; Keller, A. *J. Polym. Sci., Polym. Phys. Ed.* **1982**, *20*, 1733–1748.
(19) Wunderlich, B. *Macromolecular Physics*; Academic Press: New York, 1976; Vol. 2, pp 16–35.
(20) (a) Barham, P. J. *J. Mater. Sci.* **1984**, *19*, 3826–3834. (b) Organ, S. J.; Barham, P. J. *J. Mater. Sci.* **1991**, *26*, 1368–1374.
(21) (a) Horowitz, D. M.; Clauss, J.; Hunter, B. K.; Sanders, J. K. M.; George, N. International Patent Application PCT/GB93/02014, 1993.

Scheme 1



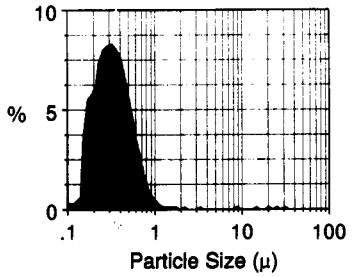


Figure 1. Particle size volume distribution for CTAB-coated artificial PHB granules, as determined by laser light scattering. The median of the distribution function was 0.3–0.4 μm.

presence of mobile, amorphous material within the artificial PHB particles. The crystallization properties of the artificial granules provide strong evidence for the competence of the nucleation model to explain PHB behavior *in vivo*.

Results

Preparation of Artificial PHB Granules. Artificial, amorphous PHB granules were prepared as shown in Scheme 1. First, a chloroform solution of PHB was emulsified ultrasonically with 20 volumes of an aqueous surfactant (e.g. 50 mM cetyltrimethylammonium bromide, CTAB). The resulting opaque emulsion contained PHB in chloroform solution, as confirmed by ^{13}C-NMR spectroscopy. The organic solvent was removed from the emulsion by (a) heating (75 °C for 90 min); (b) stirring in air (40 h); or (c) dialysis against additional aqueous surfactant. The translucent suspension that formed contained amorphous, surfactant-coated PHB particles, which were similar to native granules by a variety of criteria, as described below. This general procedure could be used to make artificial granule suspensions from PHB, PHBV copolymers (various compositions), poly-3-hydroxyoctanoate (PHO), and several other PHAs. Dichloromethane, but not 1,2-dichloroethane, could also be used as a solvent for PHB to produce amorphous granules; water is the only solvent identified thus far that may be used for the continuous phase. A variety of surfactants could be used to prepare granules, which by the criterion of ^{13}C-NMR spectroscopy, contained amorphous PHB (see Chart 1). These included a number of cationic detergents, of the quaternary amine family; several common anionic detergents, including sodium dodecyl sulfate (SDS) and the bile acid salts sodium cholate and deoxycholate; common fatty acid salts (soaps); and one nonionic detergent. Omission of the surfactant from the system resulted in rapid flocculation and crystallization of the PHB following removal of the organic solvent. While ultrasonic treatment was the preferred method of emulsification, a mechanical homogenizer was also partially effective. Ultrasonic treatment resulted in some reduction of the average polymer molecular weight (M_w), from 690 000 to 140 000–330 000.

Artificial granules could be concentrated by centrifugation, which was usually carried out in two rounds of 30 min each at

Chart 1

Cationic Detergents

Benzalkonium Cl
Benzethonium Cl
Benzyldimethyldodecylammonium Br
Benzyldimethylhexadecylammonium Br
Benzyldimethyltetradecylammonium Br
Cetyldimethylethylammonium Br
Cetylpyridinium Cl
Cetyltrimethylammonium Br
Dodecyltrimethylammonium Br
Methylbenzethonium Cl
Tetradecyltrimethylammonium Br

Nonionic Detergent

Sorbitan Monopalmitate

Anionic Detergents

Sodium Cholate
Sodium Deoxycholate
Sodium Dioctylsulfosuccinate
Sodium Dodecyl Sulfate
Sodium Sarkosyl

Soaps

Sodium Laurate
Sodium Myristate
Sodium Palmitate
Sodium Stearate

8000 and 33000*g*. Provided the granules were promptly resuspended in water, centrifugation did not appear to promote crystallization, in contrast to the behavior reported for native granules.[10a]

Artificial Granule Size and Morphology. Artificial granules were examined by several techniques to determine their size and morphological characteristics. Suspensions of granules prepared using CTAB and other surfactants were examined by laser light-scattering (Figure 1). The median particle diameter for the CTAB-coated granules estimated by this technique was 0.3–0.4 μm. Variables such as the surfactant concentration during emulsification, the duration of ultrasonic treatment, and the nature of the surfactant did not exercise any pronounced effect on the particle size distribution of the granules.

In addition, dilute suspensions of CTAB-coated granules were air-dried onto holey carbon films and examined by transmission electron microscopy (Figure 2). The granules appeared as somewhat deformable microspheres with typical diameter 0.1–0.3 μm. Similar results were obtained by fluorescence microscopy. Artificial granules were stained with the dye Nile Red,[22] a selective histological stain for PHB and other lipid inclusions. The granules were visible as red-orange microspheres, fluorescent at >580 nm (data not shown). Comparison with similarly stained native granules contained in whole *A. eutrophus* cells showed no significant difference in size or morphology. It has been reported that solid fatty inclusions do not color with this dye,[23] and thus the staining of artificial granules is consistent with their containing amorphous, elastomeric PHB.

Granule Structure and Molecular Mobility. Artificial granules prepared using CTAB were collected from aqueous suspension by centrifugation at 8000*g* and applied as a paste to a glass X-ray slide. Both whole, PHB-rich cells of *A. eutrophus* (Figure 3a) and artificial granules (Figure 3c) show only an amorphous halo when viewed by WAXS. When PHB is isolated from whole cells

(22) (a) Greenspan, P.; Mayer, E. P.; Fowler, S. D. *J. Cell. Biol.* **1985**, *100*, 965–973. (b) Ostle, A. G.; Holt, J. G. *Appl. Environ. Microbiol.* **1982**, *44*, 238–241.

(23) Cain, A. J. *Biol. Rev. Cambridge Philos. Soc.* **1950**, *25*, 73–112.

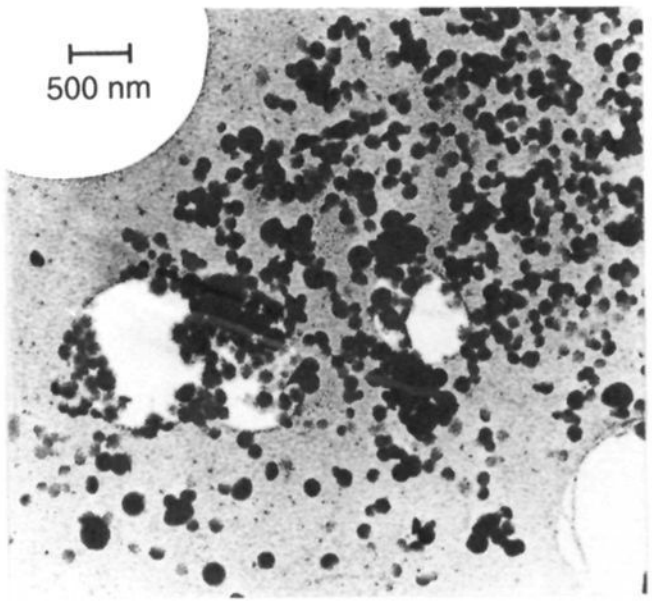


Figure 2. Transmission electron micrograph of CTAB-coated artificial PHB granules. Artificial granules appear as small dark spheres; large circles are holes in the carbon film support.

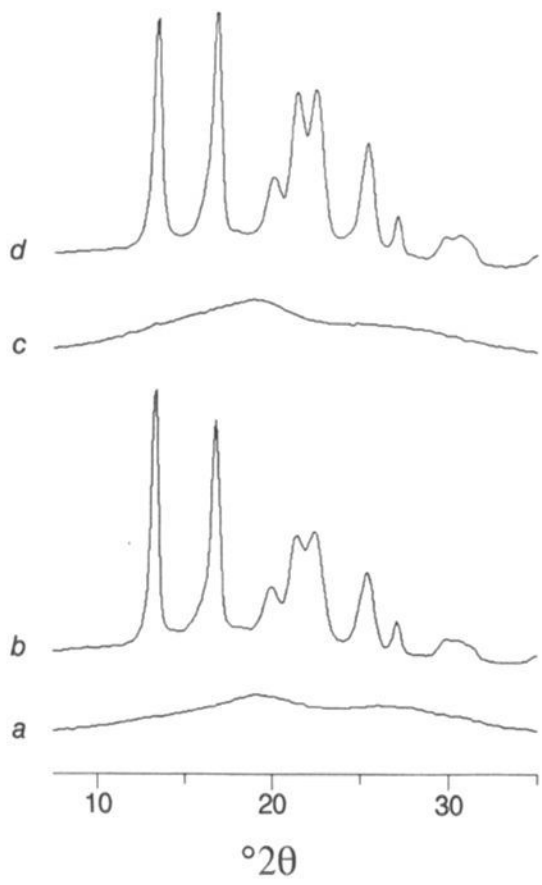


Figure 3. Wide-angle X-ray scattering (WAXS) patterns for (a) whole, PHB-rich cells of *A. eutrophus*; (b) isolated, crystalline PHB powder; (c) artificial, amorphous PHB granules; and (d) artificial granule paste dried and annealed overnight at 125 °C.

by the commercial aqueous method, the polymer undergoes ready crystallization. Thus the isolated PHB sample in Figure 3b shows a series of sharp peaks in its diffraction pattern, indicative of a crystallinity of 69%. Air drying of the granule paste sample in Figure 3c likewise results in the onset of crystallization, presumably due to the destabilization of the protective surfactant coating. On annealing overnight at 125 °C, the artificial granule paste forms a film of 73% crystallinity (Figure 3d), comparable to that of the initial powder form.

The elastomeric state of PHB granules *in vivo* was originally detected by ^{13}C-NMR spectroscopy. Elastomeric polymers at temperatures well above T_g show sharp NMR resonances, whereas the resonances derived from crystalline solids are extremely broad (>1000 Hz) and are thus invisible by conventional solution techniques. Aqueous suspensions of artificial, CTAB-coated granules and whole, PHB-rich cells of *A. eutrophus* were examined in parallel by NMR at various temperatures from 30–90 °C. The spectra obtained (Figure 4) show strikingly similar mobility properties across the entire temperature range. The NMR spectra also reveal the notable absence of residual chloroform from the

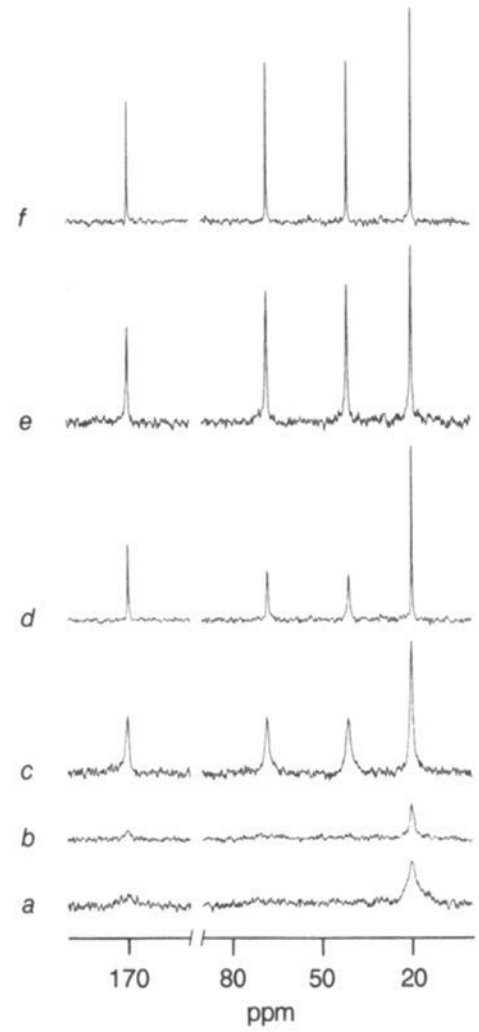


Figure 4. Natural abundance 100-MHz ^{13}C-NMR spectra of whole, PHB-rich cells a *A. eutrophus* and artificial, amorphous PHB granules. Cells or granules were collected by centrifugation and resuspended in D_2O. Samples a, c, and e are whole cells at 30, 50, and 90 °C, respectively; samples b, d, and f are artificial granules at 30, 50, and 90 °C. Signals at δ 170, 68, 41, and 20 ppm represent the PHB carbonyl, methine, methylene, and methyl resonances, respectively.

artificial granules;[24] furthermore, only a trace of surfactant can be seen. It should be noted that 70% crystalline PHB powder has no NMR spectrum at these temperatures, as the amorphous domains of the polymer are sufficiently small and constrained to prevent their giving rise to sharp lines. Determinations of the NMR relaxation time constants T_1 and T_2, described elsewhere,[25] also reveal a remarkable similarity between native and artificial granules over a range of temperatures.

Granule Density Analysis. The densities of native and artificial granules were determined in parallel by Nycodenz density gradient centrifugation. Crude native PHB granules were isolated by a gentle lysozyme/ultrasound treatment from *A. eutrophus* grown under nutrient limitation; the native granules were then purified on sucrose step gradients. Suspensions of purified native granules and SDS-coated artificial granules were applied to linear 30–50% (wt/v) Nycodenz gradients, which were developed at 110000*g*. Gradients were fractionated and the fractions assayed quantitatively for PHB (Figure 5). The average density of artificial granules was estimated to be 1.180 g/cm^3, compared to 1.170 g/cm^3 for native granules. Both values were similar to the density of 1.176 g/cm^3 reported for pure amorphous PHB quenched from the melt.[26] By contrast, crystalline PHB powder

(24) Artificial granule suspensions prepared by the heating method were examined by GC to quantify residual chloroform at various stages in the heating process. After 60 min at 75 °C, residual chloroform had been reduced to <13 ppm (i.e. <0.5% wt/wt compared to PHB). After 90 min at 75 °C, chloroform was no longer detectable in the suspension (detection limit 1–2 ppm), but extrapolation from earlier values would indicate the presence of 0.24 ppm (<100 ppm wt/wt on PHB).

(25) Shaw, G. L.; Melby, M. K.; Horowitz, D. M.; Keeler, J.; Sanders, J. K. M. *Int. J. Biol. Macromol.* **1994**, *16*, 59–63.

(26) Bauer, H.; Owen, A. J. *Colloid Polym. Sci.* **1988**, *266*, 241–247.

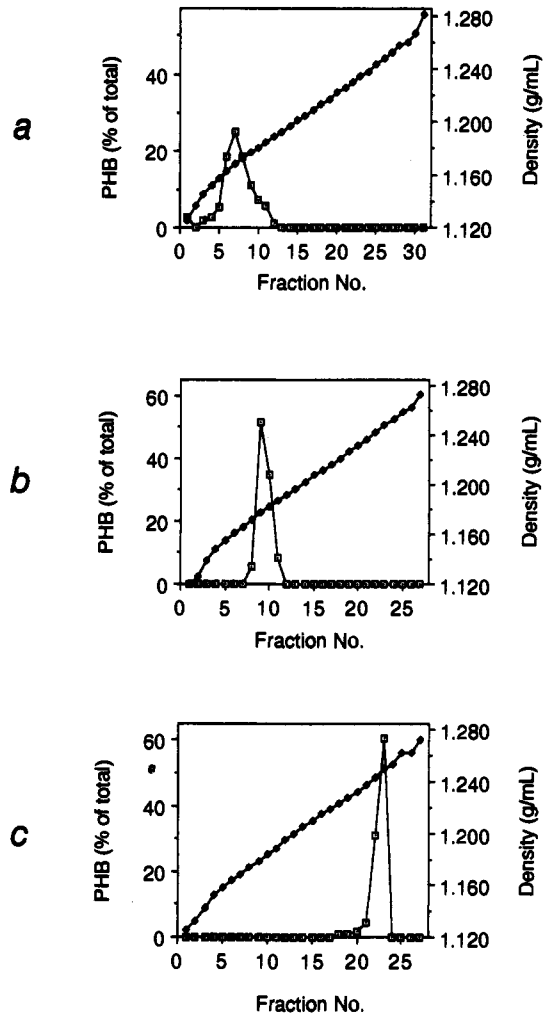


Figure 5. Nycodenz density gradient profiles for (a) isolated, native PHB storage granules from *A. eutrophus*; (b) artificial, amorphous PHB granules (SDS-coated); and (c) crystalline PHB powder. Linear Nycodenz gradients (prepared from 30–50% wt/v stock solutions) were loaded with sample, developed at 110000*g*, and fractionated. Fractions were analyzed by gas chromatography for PHB (open squares); density was determined by refractometry (closed diamonds).

exhibited a density of 1.246 g/cm^3 in Nycodenz (identical to the literature value[27]).

Artificial Granule Stability. A sample of CTAB-coated artificial granules was maintained at 30 °C for a prolonged period in a sealed NMR tube containing a benzene-d_6 capillary as internal reference (Figure 6a). The sample was examined regularly by ^{13}C-NMR spectroscopy (70 °C) to detect amorphous PHB. After a period of nearly one year, there was no measurable decrease in the intensity of the four polymer resonances relative to that of the benzene internal standard (Figure 6b). Similar stability was observed with granules prepared using the anionic surfactants SDS, sodium sarkosyl, sodium deoxycholate, and sodium dioctylsulfosuccinate (data not shown).

Although artificial amorphous granules appeared to be stable indefinitely when not perturbed, stability was absolutely dependent upon the presence of the surfactant coating. When a sample of CTAB-coated PHB granules was dialyzed against a large volume of water to remove the surfactant, flocculation and crystallization rapidly ensued. No polymer signals were visible in the ^{13}C-NMR spectrum of this sample following dialysis (Figure 6c). However, inclusion of 1 mM CTAB in the dialysis buffer was sufficient to protect the granules from denaturation (data not shown).

(27) Barker, P. A.; Mason, F.; Barham, P. J. *J. Mater. Sci.* **1990**, *25*, 1952–1956.

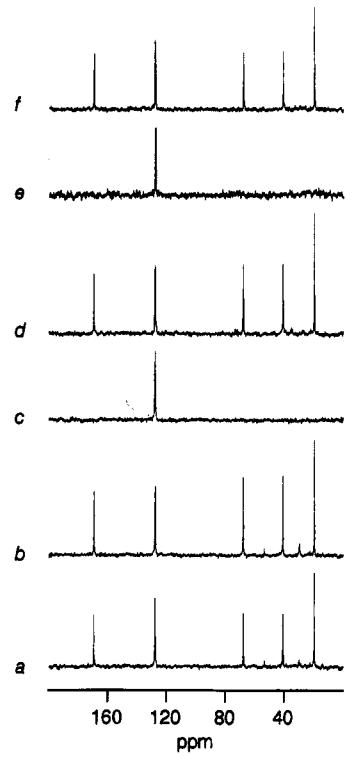


Figure 6. Natural abundance 100-MHz ^{13}C-NMR spectra, at 70 °C, of aqueous suspensions (D_2O or H_2O) of various artificial granule preparations. The peak in each spectrum at δ 128 ppm is derived from a sealed benzene-d_6 capillary, which served as an internal intensity standard. Spectrum a was obtained from a freshly prepared sample of CTAB-coated, artificial PHB granules; spectrum b was obtained from the identical sample after 11 months' incubation at 30 °C. Spectrum c was obtained from a sample of CTAB-coated, artificial amorphous granules dialyzed exhaustively against distilled water. Spectrum d was collected from a sample of artificial amorphous PHB granules prepared using the detergent sodium cholate. Dialysis of these granules against distilled water causes crystallization and loss of NMR signals (e). Addition of solubilized soy phospholipids prior to dialysis protects the granules from crystallization, leaving the NMR spectrum intact (f).

Phospholipid-Coated Granules. The readiness with which water-soluble surfactants could be removed from the granules suggested that the granule coating could be replaced by exchange with other amphipathic substances. Accordingly, artificial granules coated with sodium cholate (NMR spectrum, Figure 6d) were collected by centrifugation and resuspended in an aqueous buffer containing soy phospholipids solubilized by sonication in a buffer containing sodium cholate (2%). A variety of lipid concentrations, from 0 to 40 mg/mL, were used. Granules resuspended in lipid/detergent cocktail were then dialyzed exhaustively against phosphate buffer containing Amberlite XAD-2 to remove the cholate detergent. Inclusion of soy phospholipids at concentrations of 10 mg/mL or higher was found to protect PHB NMR signals from loss during dialysis (Figure 6f); in the absence of added lipid these signals were completely lost (Figure 6e), as with the CTAB-coated granules above.

Native-Granule-Associated Phospholipids. Lipids extracts were prepared from whole, PHB-rich cells of *A. eutrophus* and from sucrose density gradient-purified native granules. Analysis of the two samples by two-dimensional TLC revealed the same spectrum of four phospholipids in each. Three were apparently identical to phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol. The fourth minor component (R_{f1} – 0.33, R_{f2} – 0.34), which was ninhydrin-positive and stained with the Dittmer phosphate reagent, has not been identified.

Artificial Granules from Polymer Mixtures. Artificial granules were prepared as above from a chloroform solution containing 2.5% (wt/v) of PHB and 2.5% (wt/v) of poly-3-hydroxybutyrate-

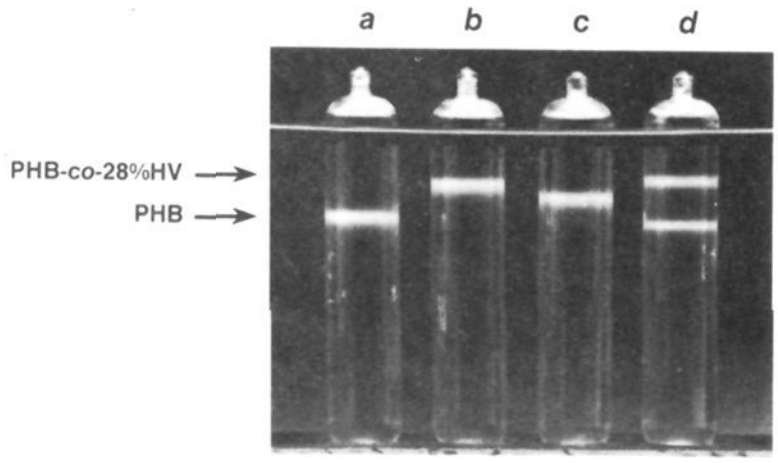


Figure 7. Nycodenz density gradients (prepared from 30–50% wt/v stock solutions) of various SDS-coated artificial amorphous granule samples. Gradient a was loaded with granules prepared from PHB homopolymer (ρ = 1.18 g/cm^3), while gradient b contained granules made from poly-3-hydroxybutyrate-*co*-28%-3-hydroxyvalerate (PHB-*co*-28%HV, ρ = 1.16 g/cm^3). Gradient c was loaded with granules made from a chloroform solution containing PHB and PHB-*co*-28%HV in equal proportions. Gradient d was loaded with a mixture of separately prepared PHB and PHB-*co*-28%HV granules. Note that in gradient c a single band of homogeneous density was observed ($\rho \approx$ 1.17 g/cm^3), indicating the coexistence of PHB and PHB-*co*-28%HV, two polymers that are immiscible in the melt, within individual artificial granules. In a mixture of PHB and PHBV granules (d), two distinct bands occur, showing that the results in gradient c are not simply due to aggregation of granules derived from two separate populations.

co-28%-hydroxyvalerate, two polymers that are incompatible in the melt.[28] Following organic solvent removal, the granules were concentrated by centrifugation and examined on Nycodenz density gradients, in parallel with samples of pure granules obtained from each polymer separately (Figure 7). It was found that the mixed polymer granules formed a single homogeneous population (Figure 7c), with density intermediate between that of the two pure polymer granule samples. In a control experiment to exclude the possibility of granule aggregation, it was shown (Figure 7d) that a mixture of separately prepared PHB and PHBV granules displayed a clearly bimodal density distribution. A paste of mixed polymer granules, which was completely amorphous by WAXS (data not shown), formed a 60% crystalline film on drying and annealing at 125 °C.

Discussion

Artificial Granules and the Nucleation Model. The foregoing results demonstrate that isolated, crystalline PHB can be simply reconstituted into an aqueous suspension of amorphous polymer particles, which are virtually indistinguishable from native PHB storage granules. The procedure used to prepare these artificial granules is effective apparently because (1) at the point at which the polymer is precipitated from chloroform solution, it is already in the form of particles that are too small to nucleate spontaneously and (2) the ionic detergent used in the emulsification provides a robust coating for the PHB, both protecting the granules from heterogeneous nucleation catalysts and preventing granule coalescence through electrostatic repulsion.

A variety of techniques was used to characterize the artificial granules. Electron micrographs indicate that the size range of the artificial granules, 0.1–0.3 μm, is essentially the same as that of native storage granules from *A. eutrophus*, a conclusion that is confirmed by light scattering and optical fluorescence microscopy. Wide-angle X-ray scattering and ^{13}C-NMR data further demonstrate that the granules contain PHB in a wholly amorphous state, with molecular mobility essentially identical to that of the native polymer. Buoyant density measurements provide further confirmation of the similar physical state of native and artificial granules.

(28) Hammond, T. Personal communication.

The PHB used in these experiments was of extremely high purity (99.9%), and thus models in which endogenous plasticizers or other auxiliary factors are required to keep the polymer amorphous *in vivo* are explicitly disproved. The only cofactor required to maintain the polymer in an amorphous state in the artificial system is an amphiphatic species capable of coating the polymer granules. Artificial granules may be prepared using any of a wide array of synthetic surfactants, indicating that no specific surfactant–polymer interaction is responsible for the amorphous state of the polymer.

In one recently proposed alternative to the nucleation model, a small amount of hydrogen-bonded water in the native granule interior maintains the polymer in a hypothetical β-conformation that is incapable of crystallizing.[14] Infrared spectroscopy and other measurements do support the presence of some water (≤5%) associated with both native granules[14b] and artificial granules (data not shown). These methods cannot distinguish, however, between water entrapped in pockets within or between the granules and water that is actually PHB-bound. In any case, no model system has been reported to date in which the polymer remains amorphous through formation of a complex with water. On the other hand, our results strongly support the kinetic nucleation model. It is especially noteworthy that removal of the surfactant from the artificial granules by dialysis results in ready crystallization of the polymer. Surfactant removal allows the coalescence of the artificial granules into larger masses (which have higher nucleation frequencies) and/or allows the polymer access to nucleating contaminants in surfaces. Small amounts of surfactant added to the dialysis buffer completely protect the granules from crystallization. None of these observations can be accommodated by the thermodynamic water model.

The stability of the artificial granules is far greater than that of native granules or of most other polymer droplets studied. The extraordinary stability of the artificial amorphous granules at 30 °C is, however, entirely in keeping with the >1000 year half-life predicted by the kinetic model. Even at 50 °C, where the spontaneous nucleation rate constant for PHB is maximal,[20b] the predicted half-life for granule crystallization is still on the order of at least 100 years. Indeed, artificial granules that were repeatedly heated from 30 to 70 °C during the course of one year for NMR experiments showed no measurable loss of signal. Thus the bacterial polyesters appear to form a class of substances where no significant solidification of amorphous droplets occurs over the entire range of temperatures where crystallization could occur.

Although slow crystallization has previously been seen with other polymer droplets,[18] in all cases (with the possible exception of isotactic polybutene-1)[18e] the amorphous droplets could withstand only a finite degree of undercooling. Critical solidification usually occurred at a point 50–100 °C below the bulk T_m. The comparatively high stability of artificial amorphous PHB granules may be due to (1) the absence of solid catalyst residues or other nucleating impurities from the bacterial polyester, resulting in an apparently lower spontaneous nucleation rate; (2) the unique protection from solid surfaces, the suspending medium,[29] and other potential nucleants afforded by the surfactant coating utilized in the current work; and/or (3) the very small particle size achieved with the PHB granules. Experiments are underway to distinguish among these possibilities.

Native Granule Proteins. The nature of the protective surface coating of native PHB storage granules is the subject of continuing

(29) Interfacial nucleation by the suspending medium has been invoked to explain the crystallization behavior of droplets of several polymers and other materials and is a possibility in the PHB system. If water itself can serve as a nucleating agent for the polymer, it could explain why amorphous PHB particles are ever observed to crystallize, as in the dialysis experiment or in the commercial harvesting process. For other cases see ref 18d and also the following: Frensch, H.; Jungnickel, B.-J. *Colloid Polym. Sci.* **1989**, *267*, 16–27. Cordiez, J. P.; Grange, G.; Mutaftschiev, B. *J. Colloid Interface Sci.* **1982**, *85*, 431–441. Grange, G.; Lévis, A.; Mutaftschiev, B. *J. Colloid Interface Sci.* **1986**, *109*, 542–551. Rasmussen, D. H.; Javed, K.; Appleby, M.; Witowski, R. *Mater. Lett.* **1985**, *3*, 344–348.

interest. The presence of protein, in particular an active PHB polymerase, in association with PHB storage granules has been demonstrated in a number of organisms;[30] in *A. eutrophus* it has been demonstrated that the bound PHB polymerase resides exclusively at the granule surface.[31] Detailed analyses of the granule-bound proteins in *Chromatium vinosum* D and *Pseudomonas oleovorans* have revealed the presence of one or more polymerases along with lower molecular weight proteins of unknown function.[30d,32] In *A. eutrophus*, there is striking evidence that a low molecular weight, granule-associated protein (the *phaK* gene product) plays a surfactant-like role in granule formation and/or stabilization.[33]

Role of Phospholipids. Data reported here represent the first detailed analysis of phospholipids associated with native PHB granules. The three phospholipids identified were found in comparable proportions in whole cell and granule lipid extracts. Cellular phospholipids in *A. eutrophus* have been analyzed previously with similar results.[34] Those authors also reported that a 1.8-fold increase in the concentration of total cellular lipids occurs during the early stages of PHB accumulation, which they attributed to the synthesis of a granule membrane. As the same phospholipids were found here in whole cells and granules, the possibility of contamination of the granules with fragments of the plasma membrane during isolation cannot be excluded. However, it appears more likely that during granule biosynthesis, phospholipids are recruited in an adventitious fashion to the hydrophobic surface of the polymer.

The experimental results reported here demonstrate that phospholipids by themselves are a chemically competent coating for the maintenance of the physical state of amorphous PHB granules. When detergent-coated granules were mixed with detergent-solubilized phospholipids and then dialyzed against a detergent-free buffer, little or no crystallization occurred. Untreated artificial granules were, on the contrary, completely susceptible to dialysis-induced crystallization. The most reasonable explanation is that, in the former case, the detergent coating is replaced during dialysis with a stabilizing phospholipid layer of some type. As it is certain that *in vivo* proteins are major components of the granule coating, it might be supposed that the role of lipids in the native system is to fill in gaps that occur in the coating between the various granule-associated polypeptides.

Applications. Notwithstanding the remarkable stability of the artificial granules in suspension, granule pastes crystallize easily on drying and annealing to form films of high crystallinity. The usefulness of these techniques to the preparation of biodegradable PHA coatings is being actively investigated. It is of additional interest that polymers that are otherwise incompatible in the melt (such as PHB and PHB-*co*-28%HV) are capable of coexisting in an amorphous state within individual artificial granules, as shown by density analysis, although there are likely to be phase boundaries within individual granules.[35] These mixed polymer granules may be useful in coatings or in studies of nucleation in binary polymer systems.

Conclusion. The preparation of amorphous, biomimetic granules of poly-3-hydroxybutyrate (PHB) reported here provides strong support for the recently proposed nucleation model for the physical state of polyhydroxyalkanoates (PHAs) *in vivo*. These artificial granules, which resemble native storage granules in many important respects, constitute a remarkably stable amorphous form of the polymer and have potentially significant practical applications. The results also suggest an *in vivo* model in which amphipathic lipids are recruited adventitiously to the granule surface during PHB biosynthesis and play a role in granule stabilization.

Experimental Section

General. Aqueously purified PHB (M_w 690 000; M_w/M_n 2.9; 99.9% pure) and PHB-*co*-HV[36] were obtained from Zeneca Bio Products, Billingham, England. *Alcaligenes eutrophus* H16 tron, a glucose-utilizing variant of the wild type, was obtained from Zeneca and cultured as described elsewhere to promote PHB accumulation.[25] Detergents, phospholipid standards, soy phosphatidylcholine (type II-S, containing 10-20% phosphatidylcholine, remainder other phospholipids), lysozyme, and Nycodenz (5-(*N*-(2,3-dihydroxypropyl)acetamido)-2,4,6-triiodo-*N*,*N'*-bis(2,3-dihydroxypropyl)isophthalimide) were all obtained from Sigma. Cellulose dialysis tubing was obtained from Medicell International (London) and had a molecular weight cutoff of 12 000–14 000. Nile Red was prepared from commercial Nile Blue A by acidic hydrolysis.[37] Sonications were performed at a frequency of 20 kHz using a Heat Systems-Ultrasonics W-375. Particle size measurements were made using a Malvern MS 20 laser particle sizer. Polymer molecular weight determinations by gel-permeation chromatography (GPC) and GC chloroform analyses were carried out by Zeneca Bio Products.

Native Granule Preparation. All manipulations were performed at 4 °C unless otherwise indicated. PHB-containing *A. eutrophus* cells grown under nutrient limitation (0.75-L culture) were centrifuged (3500*g*, 30 min) and resuspended in 15 mL of buffer A (50 mM sodium phosphate, pH 8.0) containing 1 mM EDTA. Hen egg-white lysozyme (20 mg) was added, and the suspension was incubated at 37 °C for 45 min. The suspension was then cooled to 4 °C and sonicated (50-W power) for 3 min on an ice–water bath. Sucrose gradients were prepared from 1.00, 1.25, 1.50, 1.75, and 2.00 M sucrose (35 mL total), and 4 mL of sonicated cell suspension was applied to each gradient. The gradients were centrifuged in a Beckman SW28 swinging bucket rotor at 100000*g* for 1 h (20 °C); native granules were collected at the 1.25–1.50 M interface (density 1.16–1.19 g/cm^3). Native granules were removed from the gradients, dialyzed overnight against buffer A, and stored at 4 °C.

Artificial Granule Preparation. In a typical procedure, a solution of PHB in chloroform (1–10 mL, 5% wt/v) was placed in the bottom of a heavy-walled glass tube or beaker, and 20 volumes of an aqueous solution of cetyltrimethylammonium bromide or other detergent (5–100 mM) was added. The two layers were then emulsified using a probe-type ultrasonicator (1–3 min at 200-W power). Heating of the sample was controlled with an ice–water bath. Chloroform was then removed from the emulsion by one of three techniques: (a) heating; (b) stirring in an open vessel; or (c) dialysis against aqueous surfactant. All operations were carried out in a ventilated fume hood. In method A, the emulsion was maintained at 75 °C for 90 min in an uncovered, magnetically stirred flask. In method B, the emulsion was stirred at ambient temperature in a wide-mouth vessel for 40 h, adding water as necessary to maintain constant volume. In method C, the emulsion was dialyzed exhaustively over 48 h against additional aqueous detergent of concentration equal to that of the original solution. The resulting granule suspension was stored at room temperature. Granules could be concentrated by centrifugation, which was conveniently done in two rounds of 30 min each at 8000*g* and 33000*g*, respectively (20 °C).

NMR Studies. Artificial granules were collected by centrifugation as described above, immediately resuspended in D_2O (0.5 mL), and

(30) (a) Haywood, G. W.; Anderson, A. J.; Dawes, E. A. *FEMS Microbiol. Lett.* **1989**, *57*, 1–6. (b) Merrick, J. M.; Doudoroff, M. *Nature* **1961**, *189*, 890–892. (c) Fukio, T.; Yoshimoto, A.; Matsumoto, M.; Hosokawa, S.; Saito, T.; Nishikawa, H.; Tomita, K. *Arch. Microbiol.* **1976**, *110*, 149–156. (d) Liebergesell, M.; Schmidt, B.; Steinbüchel, A. *FEMS Microbiol. Lett.* **1992**, *99*, 227–232.

(31) Gerngross, T. U.; Reilly, P.; Stubbe, J.; Sinskey, A. J.; Peoples, O. P. *J. Bacteriol.* **1993**, *175*, 5289–5293.

(32) Fuller, R. C.; O'Donnell, J. P.; Saulnier, J.; Redlinger, T. E.; Foster, J.; Lenz, R. W. *FEMS Microbiol. Rev.* **1992**, *103*, 279–288.

(33) Disruption of the *phaK* gene results in the production of one large PHB granule in each cell, while its overexpression results in the formation of a large number of minigranules: Steinbüchel, A. Personal communication.

(34) Thiele, O. W.; Dreysel, J.; Hermann, D. *Eur. J. Biochem.* **1972**, *29*, 224–236.

(35) We have also prepared artificial granules from a combination of PHB and poly-3-hydroxyoctanoate (PHO), another bacterial polyester. There was strong evidence for phase separation within these granules. As with the PHB/PHBV granules, density analysis showed that individual granules contained both polymers. In this case, however, the glass transition of the PHO, which occurs some 40 °C lower than that of PHB, could be observed directly by differential scanning calorimetry (DSC). The T_g of PHO contained in the PHB/PHO mixed polymer granules was found to be −36 °C, essentially the same as in bulk amorphous PHO, pure PHO artificial granules, or PHO contained in quenched co-melts with PHB (which showed two independent glass transitions at −36 and 5 °C): Horowitz, D. M.; Sanders, J. K. M. Unpublished results.

(36) Holmes, P. A.; Lim, G. B. European Patent Application 145 233, 1985.

(37) Thorpe, J. F. *J. Chem. Soc.* **1907**, *91*, 324–336.

transferred to a 5-mm NMR sample tube fitted with a sealed capillary containing benzene-d_6 (50 μL). ^{13}C-NMR spectra were recorded at 100.6 MHz on a Bruker AM-400 NMR, utilizing an acquisition time of 0.279 s and a recycle time of 5 s. 16K data points were collected, and an exponential line-broadening factor of 20 Hz was applied prior to Fourier transformation. Each spectrum is the result of acquiring 200–800 transients. Sample temperatures were varied from 30 to 90 °C. The relative fraction of the particles in a mobile, amorphous state was estimated from the intensity of the methyl carbon resonance (δ 20 ppm) relative to that of the benzene reference peak (δ 128 ppm) at 70 °C. Whole cell NMR spectra were obtained under identical conditions using PHB-rich cell pastes (50–100 mg wet) resuspended in D_2O (0.5 mL).

Wide-Angle X-Ray Scattering. Artificial granules were collected by centrifugation (8000*g*, 30 min, 20 °C), applied as a paste (*ca.* 50% solids, 1-mm-thick film) to a glass X-ray slide, and analyzed immediately by wide-angle X-ray scattering (WAXS) using a Philips PW1710 diffractometer, using Cu Kα radiation (λ = 1.5418 Å). WAXS measurements were performed periodically as the sample dried in air and following thermal annealing treatments. Percent crystallinity was estimated using a curve-fitting computer program furnished by Prof. Brian K. Hunter (Queens University, Ontario) or by cutting and weighing.

Granule Density Analysis. Equal volumes (2.5 mL each) of five aqueous solutions (30, 35, 40, 45, 50% wt/v) of Nycodenz were layered into 13-mL ultracentrifuge tubes. Sample (0.1 mL, containing *ca.* 10 mg of PHB) was added, and the tubes were sealed and placed in a horizontal position for 45 min to linearize the gradients. The gradients were then centrifuged for 2 h at 110000*g* (20 °C) in a Beckman 70.1 Ti fixed-angle ultracentrifuge rotor. Fractions of 0.5 mL were collected from each gradient. The refractive index of the fractions was read with an Abbé refractometer (Bellingham and Stanley) and the density calculated using the literature formula.[38] The fractions were freeze-dried, and the PHB content of each was determined by the published propanolysis method[39] using a Carlo Erba Strumentazione 4130 gas chromatograph equipped with a 25-m BP5 fused silica capillary column (SGE).

Fluorescence and Electron Microscopy. CTAB-coated artificial granules (0.25 mg/mL) or 5-fold diluted cultures of *A. eutrophus* were stained with 1 μg/mL Nile Red. Aliquots were pipetted onto glass slides and allowed to dry overnight. The stained samples were visualized using a Zeiss Axiavert 35M inverted fluorescence microscope with an excitation wavelength of 535 nm and an emission wavelength of >580 nm. For transmission electron microscopy, a suspension of CTAB-coated artificial granules in water (25 μg/mL) was spotted onto a holey carbon film and allowed to dry overnight at ambient temperature and pressure. The granules were examined using a JEOL JEM-2000EX electron microscope operating at 200 kV. Sample temperature was maintained at −167 °C by use of a liquid nitrogen cold-stage.

Long-Term Stability. Artificial granules were prepared as above (by the heating method) using CTAB, SDS, sodium deoxycholate, sodium sarkosyl, or sodium dioctylsulfosuccinate as surfactants (each at 50 mM). The granules were centrifuged, resuspended in D_2O (0.5 mL), and sealed into NMR tubes fitted with benzene-d_6-containing capillaries. The samples were maintained at 30 °C in an incubator for several months and periodically removed to obtain ^{13}C-NMR spectra at 70 °C.

Removal of Surfactant by Dialysis. Two identical samples of CTAB-coated artificial granules were concentrated as above by centrifugation to *ca.* 10% solids in H_2O (0.5 mL). Sample A was dialyzed overnight against 1 mM aqueous CTAB (1 L), while sample B was dialyzed in parallel against distilled water. The two samples were then examined by ^{13}C-NMR spectroscopy at 70 °C to detect amorphous PHB.

Phospholipid Analysis. Whole, PHB-rich cells of *A. eutrophus* or native *A. eutrophus* granules prepared as above were collected by centrifugation and resuspended in 2:1 (v/v) methanol/chloroform (120 mL). The suspensions were disrupted by ultrasonication (5 min). Water (65 mL) and then chloroform (40 mL) were added, and the two phases were allowed to separate. The organic phase containing PHB and lipids was evaporated to dryness *in vacuo*. The residue was taken up again in chloroform (4 mL), and methanol (25 mL) was added. The precipitate, consisting mainly of PHB, was collected by centrifugation and then redissolved, reprecipitated, and discarded. The combined supernatants from the two rounds of centrifugation were evaporated to dryness *in vacuo* to give the partially purified lipid extract.

The granule and cellular lipid fractions from above were analyzed by two-dimensional thin-layer chromatography on silica gel plates. The plates were developed first with 65:30:3:1 chloroform/methanol/water/40% aqueous NH_3 (system 1), dried *in vacuo*, and then developed in the second dimension with 75:25:8:3 chloroform/methanol/acetic acid/water (system 2). Lipids were visualized by charring with aqueous $(NH_4)_2SO_4$ (20% solution) or by staining with ninhydrin (0.25% in acetone) or Dittmer phosphate reagent[40] and then identified by comparison with authentic phospholipid samples.

Preparation of Phospholipid-Coated Artificial Granules. Lipid-coated artificial granules were prepared by replacement of the detergent coating of conventional granules using detergent-solubilized phospholipids. Soy phosphatidylcholine was purified by a literature method[41] and stored at −20 °C as a chloroform solution at *ca* 333 mg/mL (0.1 mg/mL BHT preservative). A 2.4-mL portion of this solution was evaporated in a heavy-walled glass test tube using a stream of dry nitrogen. Diethyl ether (5 mL) was added, and the sample was again dried with nitrogen. The lipids were then dried *in vacuo* for several hours, yielding 767 mg of yellow gum. A solution of sodium cholate (2% wt/v) in buffer A (18.4 mL) was added, and the sample was sonicated at 20 kHz, power output 50 W, for 5 min on an ice–water bath. The product, which was reserved, was a clear yellow homogeneous suspension containing 40 mg/mL soy lipids.

Artificial granules were prepared by the above method using 50 mM sodium cholate as the detergent. The cholate-coated granule suspension (30 mL) was centrifuged first at 8000*g* for 30 min (20 °C), and then the supernatant was recentrifuged at 33000*g* for 45 min (20 °C). Pellets from the two rounds of centifugation were resuspended in buffer A (0.5 mL total) containing a total of 2% sodium cholate plus 0–40 mg/mL solubilized soy lipids from above. The samples were transferred to cellulose dialysis bags and dialyzed exhaustively over a period of 24 h at room temperature against large volumes of buffer A containing 25 g/L Amberlite XAD-2 resin. ^{13}C-NMR spectra were then obtained for all samples at 70 °C, using a benzene-d_6 capillary as internal reference.

Acknowledgment. Financial support for this research was received from Zeneca Bio Products, the National Science Foundation (predoctoral fellowship to D.M.H.), the American Friends of Cambridge University, and the Cambridge Overseas Trust. We thank Joachim Clauss, Andrew Eddy, Grant Grieve, Timothy Hammond, Brian Hunter, Melissa Melby, Yair Shachar-Hill, Graeme Shaw, Steve Wylie, and especially Gerald Smith for advice, assistance, and materials.

(38) Rickwood, D. Properties of Iodinated Density-Gradient Media. In *Iodinated Density Media; A Practical Approach*; Rickwood, D., Ed.; IRL: Oxford, 1983; pp 1–21.

(39) Riis, V.; Mai, W. *J. Chromatogr.* **1988**, *445*, 285–289.

(40) Dittmer, J. C.; Lester, R. L. *J. Lipid Res.* **1964**, *5*, 126–127.

(41) Eytan, G. D. Reconstitution of Membrane Function with Liposomes. In *Techniques in the Life Sciences*; Hesketh, T. R., Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I., Tipton, K. F., Eds.; Elsevier Biomedical: County Clare, Ireland, 1982; Vol. B416.