Poly(3-hydroxybutyrate) (PHB) is well-known as a high molecular weight homopolymer of R-3-hydroxybutyrate which accumulates in storage granules within the cytosols of certain bacteria. Escherichia coli does not amass these granules; however, small amounts of low molecular weight PHB (<0.02% of dry weight) have been found in the plasma membranes in complexes with calcium polyphosphate; the complexes serve as voltage-activated calcium channels. Here we report that polyphosphate-complexed PHB is only a minor fraction of the polyester in the plasma membranes of genetically competent bacteria, complexed with calcium polyphosphate (PPi) (Reusch and Sadoff, 1988). These cPHB-polyphosphate complexes function as voltage-activated calcium channels (Reusch et al., 1995), and they have also been postulated to serve in coexport of calcium and phosphate, in DNA transmembrane transfer, and as calcium stores (Reusch and Sadoff, 1988). The association of cPHB with proteins has been reported previously in plasma (Reusch et al., 1992).

Current protocols for determining PHB in bacteria were designed to measure the high molecular weight uncomplexed polyester found in inclusion granules (Holmes, 1987); consequently, they exploit one or more of the physical and chemical properties of the bulk polymer, solubility in chloroform, insolubility in water, methanol, acetone, and ether, resistance to degradation by alkaline hypochlorite, and acid-catalyzed degradation to crotonic acid. We became aware of the inadequacies of these methods when they failed to detect cPHB in samples of bovine serum albumin that displayed strong positive reactions to polyclonal anti-PHB IgG (Reusch et al., 1992). Subsequently, cPHB was extracted from bovine serum albumin with a mixed solvent system, and its identity was confirmed by chemical assay and 1H NMR (Reusch, 1992; Seebach et al., 1994). The failure of traditional procedures could in part be attributed to their insensitivity to the comparatively low concentrations of cPHB in the samples, but could also be related to changes in the physical and chemical properties of the polyester effected by its association with protein.

This study was undertaken to determine whether cPHB-protein complexes exist in Escherichia coli. We investigated several common procedures for assaying PHB in bacteria and developed a sensitive protocol for determination of cPHB. Using this improved assay and anti-PHB IgG, we detected significant quantities of cPHB in E. coli and found that the polyester is widely distributed throughout the cell, wherein it is largely associated with proteins.

EXPERIMENTAL PROCEDURES
Reagents and Strains
Organisms used in this study were E. coli strains K12, DH5α, JM101, and CA10 (kindly provided by A. Kornberg). SOB medium (Hanahan, 1987) was 2% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 20 mM MgCl2. CB medium (competence buffer) was 10 mM CaCl2, 45 mM MnCl2, 100 mM KCl, in 10 mM MES, pH 6.3. MSG medium was minimal salts M9 medium, 6.0 g of Na2HPO4, 3.0 g of KH2PO4, 0.5 g of NaCl, 1.0 g of NH4Cl in 1 liter of distilled water with 0.5% glucose. All salts used in preparation of CB medium were high purity quality (>99%) from Aldrich.

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Preparation of Cells

E. coli were cultured in SOB medium, or minimal salts M9 medium containing 0.5% glucose (MSG), at 37°C with shaking at 180 rpm to an A550 of 0.4–0.5. For J M101, MSG was supplemented with casamino acids at a final concentration of 0.2%. Cells were made genetically competent by a variation of the method of Hanahan (1983); cells in a log-phase culture in SOB medium were collected by centrifugation at low speed (800 × g) at 4°C and were suspended in one-third volume of ice-cold CB medium for 30 min. The number of live cells was determined by plating cells on SOB agar; total cells were determined by counting in a Petroff-Hauser bacteria counter (C. A. Hauser & Sons); dry weight was determined by heating washed cell pellets in a 105°C oven to constant weight.

Determination of PHB

Method 1: Methanolysis in 3% Sulfuric Acid—The procedure used was that of Braunegg et al. (1978). Briefly, a mixture of methanol acidified with H2SO4 (3% v/v) and chloroform (1:1) was added to lyophilized cells. The preparation was heated at 100°C for 4 h and cooled to ambient temperature. Water was added and the organic phase was analyzed with a Perkin Elmer AutoSystem Gas Chromatograph using a 30-m × 0.32-mm inside diameter Carbowax PEG column. Quantitation was done by evaluating peak areas using granule PHB (Aldrich) as standards.

Method 2: Hydrolysis in 2 N NaOH—The procedure of Brandl (1992) was used. Briefly, lyophilized cells were heated in 2 N NaOH at 100°C for 30 min. The cooled samples were neutralized with 2 N HCl and analyzed by HPLC as described in Method 3 below.

Method 3: Acid-catalyzed β Elimination to Form Crotonic Acid—The procedure used was an adaptation of the method of Karr et al. (1983). Concentrated sulfuric acid (0.5 ml) was added to a dry cell pellet (from 30 ml of culture) and the mixture was stirred and heated in a dry heating block (Pierce) at 92°C or other desired temperature. At specified time periods, the tube was cooled on ice, 1 ml of saturated sodium sulfate was added, and the solution was extracted 4 times with 3 ml of dichloromethane. Sodium hydroxide (100 μl of 1 N) was added to the dichloromethane extract to convert crotonic acid to crotonate, and the dichloromethane was evaporated with a stream of nitrogen. The residue was acidified to pH 2 with 3 N sulfuric acid and filtered with a 0.2-μm nylon syringe filter. The filtrate was chromatographed on an HPLC Aminex HPX-87H ion exclusion organic acid analysis column (Bio-Rad) with 0.014 N sulfuric acid as eluant at a flow rate of 1 ml/min. The crotonic acid peak was identified by comparison with known crotonic acid and by its UV absorption curve, and its identity was confirmed by mass spectrometry. Crotonic acid content was estimated by evaluation of peak area using granule PHB isolated from Azotobacter vinelandii as described previously (Reusch and Saddof, 1983), or purchased from Aldrich, as standards.

Isolation of PHB

Alkaline Hypochlorite Digestion—The cell pellet was digested with 5.25% alkaline hypochlorite (Cloro) at 37°C for 30 min. The residue was collected by centrifugation and washed sequentially with water, methanol, and acetone (2× each) and dried (Law and Slepecky, 1961). PHB was then converted to crotonic acid by heating in concentrated sulfuric acid at 92°C for 45 min. The crotonic acid was recovered and determined as described above (Method 1).

Chloroform Extraction—The cell pellet was washed sequentially with water, methanol, and acetone (2× each) and dried. The residue was extracted with 2 ml of warm chloroform (50°C) four times. The extracts were combined and the chloroform evaporated with a stream of dry nitrogen gas. PHB was then determined as above (Method 1).

Fractionation of Cells

E. coli log-phase cells (10 liters) were cultured in SOB medium to an A550 of 0.4–0.5. The cells were pelleted by centrifugation at 1800 × g, washed with 10 ml Heps buffer, pH 7.3, and then suspended in the same buffer containing 20 μg/ml RNase and 20 μg/ml DNase. The cells were broken by passage through a French pressure cell at a pressure of 18,000 p.s.i. Unbroken cells were removed by centrifugation at 7000 rpm in a GSA Rotor (Beckman) for 10 min (2×) at 4°C. The supernatant was layered over a pillow of 60% sucrose and centrifuged in a Beckman Type 50.2 Ti rotor at 35,000 rpm for 45 min at 4°C in a Beckman L5P50 Ultracentrifuge. The membranes were recovered from the sucrose interface with a J-hook and washed twice with 10 ml Heps, pH 7.4. The cytoplasm was centrifuged in a Type 50.2 Ti rotor at 200,000 g for 2.5 h (2×) to collect the ribosomal fraction (Sykes, 1971).

RESULTS

Isolation and Determination of cPHB from E. coli by Conventional Methods—Current protocols for determining high molecular weight PHB in inclusion bodies of bacteria can be divided into two categories: 1) methods in which PHB is isolated and purified before analysis; 2) methods in which PHB is determined directly in whole cells. The efficiencies of some of the most popular protocols of each class were examined to determine the most sensitive method(s) for measuring cPHB in E. coli.

First we examined two procedures of the first category: one based on the relative resistance of PHB to degradation by alkaline hypochlorite solution, and the other based on the solubility of PHB in chloroform (see “Experimental Procedures”). The alkaline hypochlorite method yielded <0.004 μg of cPHB/mg dry weight in log-phase cells and 0.14 to 0.24 μg/mg dry weight in competent cells (Table I). The recovery of cPHB by extraction with chloroform proved less efficient; this method yielded <0.001 μg of PHB/mg dry weight from log-phase cells and 0.05–0.06 μg/mg dry weight in competent cells (Table I).

Next we examined the efficiency of three procedures for direct determination of PHB in whole cells: 1) acid methanolysis with 3% sulfuric acid as described by Braunegg et al. (1978); 2) alkaline hydrolysis in 2 N NaOH as described by Brandl (1992); 3) conversion to crotonic acid by heating in concentrated sulfuric acid as described by Karr et al. (1983). As shown in Table II, the direct methods were generally more efficient in measuring cPHB. The latter two methods were about 10-fold more sensitive than the first, and the conversion to crotonic acid by hot concentrated sulfuric acid proved to be about one-third more sensitive than alkaline hydrolysis (after corrections were made for both procedures), yielding on average 0.4 μg of cPHB/mg dry weight for log-phase cells.

Further Studies of the Conversion of cPHB to Crotonic Acid in Concentrated Sulfuric Acid—The protocol that was most efficient in determining cPHB, i.e. β elimination of crotonic acid effected by concentrated sulfuric acid, was examined further. The reaction can be roughly described by Reaction 1.
Determination of cPHB from E. coli after isolation of the polyester by digestion with alkaline hypochlorite or extraction with chloroform. Cells were cultured in SOB medium to absorbance at 550 nm of 0.40 to 0.55. Competent cells (CC) were prepared by suspending the cell pellet in ice-cold CB medium.

| Method                      | Strain | Medium | PHB (µg/mg dry wt) | Temp (°C) | Time (h) |
|-----------------------------|--------|--------|---------------------|-----------|----------|
| Alkaline hypochlorite digestion\(a\) | K12    | SOB    | 0.002               |           |          |
|                             | DH5x   | SOB    | 0.003               |           |          |
|                             | DH5x CC| CB     | 0.21                |           |          |
|                             | J M101 | SOB    | 0.004               |           |          |
|                             | J M101 CC| CB    | 0.24                |           |          |
|                             | CA10   | SOB    | 0.002               |           |          |
|                             | CA10 CC| CB     | 0.14                |           |          |
| Chloroform extraction\(b\)  | K12    | SOB    | 0.001               |           |          |
|                             | DH5x   | SOB    | 0.001               |           |          |
|                             | DH5x CC| CB     | 0.08                |           |          |
|                             | J M101 | SOB    | 0.001               |           |          |
|                             | J M101 CC| CB    | 0.10                |           |          |
|                             | CA10   | SOB    | 0.001               |           |          |

- \(a\) Cell pellet was digested with 5.25% alkaline hypochlorite (1 ml/10\(^{10}\) cells) at room temperature for 30 min. The residue was washed with water, methanol, and acetone and dissolved in chloroform. After filtration, the chloroform was evaporated, 1 ml of concentrated sulfuric acid was added, and the mixture was heated at 92 °C for 45 min. Crotonic acid was then isolated and quantitated as described under "Experimental Procedures." Values are an average of 3 determinations and are corrected for incomplete conversion to crotonic acid and degradation of crotonic acid as determined for PHB granules used as standards.
- \(b\) Cell pellet was washed with methanol and acetone, and the dry residue was extracted with warm chloroform (50 °C, 4×). The extract was filtered and chloroform was evaporated. PHB content was determined by conversion to crotonic acid in concentrated sulfuric acid as in 1. Values are an average of 3 determinations.

**Table II**

Estimation of cPHB in E. coli whole cells using direct reaction protocols

Values are an average of 3 determinations and are based on standards of granule PHB. Values given in parentheses are the amounts of crotonic acid measured by the assay before correction for incomplete conversion of PHB to crotonic acid and crotonic acid degradation.

| Method                      | Strain | Medium | PHB (µg/mg dry wt) |
|-----------------------------|--------|--------|--------------------|
| Alkaline hydroylsis          | J M101 | SOB    | 0.06               |
| (2 n NaOH) \(\beta\) elimination | J M101 | SOB    | 0.3                |
| (concentrated H\(_2\)SO\(_4\)) | J M101 | SOB    | 0.4 (0.33)         |
|                             | J M101 | SOB    | 0.4 (0.20)         |
|                             | J M101 | SOB    | 0.5 (2.75)         |

\[ \text{PHB} \rightarrow \text{crotonic acid} \rightarrow Y \]

\[ X \]

**REACTION 1**

where \(X\) refers to other degradation products of PHB and \(Y\) to degradation products of crotonic acid.

This reaction is usually carried out at temperatures of 85 °C to 100 °C and typically results in 82–85% conversion of PHB to crotonic acid in 20 to 30 min (Karr et al., 1983). In our hands, the degree of conversion was found to be 85% (determined by extrapolation of the curve for degradation of granule PHB in Fig. 1A to zero). Most of the loss is attributed to conversion of PHB to other products (X) by other pathways. The maximal yield was achieved in 20 min and remained at about the same level for over 1 h (Fig. 1A). Pure crotonic acid, treated in the same manner, showed little degradation over this time period (~3%) (Fig. 1A). The net result was that a 1-µg sample of granule PHB yielded 0.82 µg of crotonic acid after 20 min at 92 °C. In contrast, the cPHB in lyophilized whole cells of E. coli J M101 produced little crotonic acid in 20 min (Fig. 1B). In this case, the yield did not level off but continued to rise for ~12 h. Over this time period, crotonic acid degradation was significant (47%) (not shown). After 12 h at 92 °C, only 0.45 µg of crotonic acid was obtained from 1 µg of granule PHB. If we assume the same efficiency for cPHB, then the log-phase J M101 cells which produced 2.20 µg of crotonic acid/mg dry weight contained ~4.9 µg of PHB (Fig. 1B and Table II). The true efficiency for cPHB is probably less (and the amount of cPHB greater), since protein-associated cPHB is more likely to be degraded by other pathways; however, this loss is at least partly offset by a reduction in crotonic acid degradation due to its slower rate of formation.

To determine the best conditions for measurement of cPHB in E. coli cells, the time required to obtain a maximal yield of crotonic acid was measured as a function of temperature. As can be seen in Fig. 2, the rate proved to be highly sensitive to...
temperature. For routine measurements, we adopted a temperature of 120 °C, at which the recovery of crotonic acid is maximal at 40 min. At this temperature, we found that only 69% of granule PHB was converted to crotonic acid (Fig. 3A). After 40 min at 120 °C, pure crotonic acid was 28% degraded (not shown), so that the yield of crotonic acid from 1 μg of granule PHB was 0.5 μg. If we again assume the same efficiency for cPHB, then the crotonic acid measured in lyophilized E. coli cells after 40 min at 120 °C represents about 50% of actual cPHB (Fig. 3B and Table II). Despite the inexactness of the measurements of cPHB, due to the lack of an appropriate standard, values obtained under the same assay conditions can provide useful comparisons of cPHB content.

cPHB in E. coli Whole Cells—Using these conditions (120 °C for 40 min), we compared cPHB levels for several strains of E. coli cells in log-phase growth (A<sub>550</sub> = 0.4). The results, shown in Table III, indicate that cPHB constitutes 0.36–0.55% of the dry weight of log-phase cells, depending on the nature of the growth medium. When cells were made competent, they produced 15–20% more cPHB (0.61–0.65% of dry weight); 10–15% of the additional polyester was isolated from the competent cell pellets, and 5% was recovered from lysed cells in the competence buffer. About 10% of this newly synthesized cPHB was chloroform-soluble. Chloroform-soluble cPHB is barely detectable in E. coli log-phase cells (Table I), and in competent cells it constitutes ~0.01% of the dry weight and ~1.5% of cPHB.

Distribution of cPHB—The cellular location(s) of cPHB in log-phase and competent cells was next determined. Cells were disrupted and separated by centrifugation into cytoplasmic and membrane fractions, and the cytoplasm was further separated by centrifugation into cytosol and ribosomal fractions. These fractions were then assayed for cPHB content. As shown in Table IV, over 90% of this highly water-insoluble polyester was found in the cytoplasm, with the largest concentration in the ribosomal fraction. The cPHB synthesized de novo during the development of competence was found mainly in the ribosomal and membrane fractions.

Identity of cPHB—The chemical assay for PHB is based on the assumption that crotonic acid is a unique product of PHB degradation in concentrated sulfuric acid. To rule out the possibility that crotonic acid could also be formed by reactions of amino acids under the assay conditions, we examined homopolymers of those amino acids whose structure suggested this capability, polythreonine, polyglutamate, polyglutamine, polymethionine, and polyarginine. None were found to produce detectable amounts (>0.001%) of crotonic acid.

The identity of cPHB in the ribosomal fraction was confirmed by 500-MHz 1H NMR spectroscopy. Although cPHB could not be extracted from the acetone-dried ribosomes by warm chloroform, small amounts of cPHB were dissolved in refluxing CHCl<sub>3</sub>:MeOH (9:1) in 1 h. This procedure had been used previously to extract cPHB from bovine serum albumin, spinach, beef heart mitochondria, and human aortal tissue (Reusch et al., 1992; Seebach et al., 1994). As shown in Fig. 4, the 1H NMR spectra of the extract includes resonances with the characteristic chemical shifts and coupling constants of the methylene and methine protons of PHB (Reusch, 1992; Seebach et al., 1994). The assignments were confirmed by selective decoupling. The methyl resonances were hidden under other signals.

Elimination of cPHB from Cell Proteins—cPHB was removed from proteins (and other macromolecules) of JM101 log-phase cells by taking advantage of the greater lability of ester bonds, as compared with amide bonds, to dilute acid hydrolysis. Cel-
The sample shows the characteristic methine and methylene protons of PHB; the methyl protons are hidden under resonances of impurities. Assignments: methylene protons split into an octet at 2.42–2.62 ppm, JAx 5.7, JBX 15.5; methine protons form a multiplet centered at 5.23 ppm. The assignments were confirmed by selective decoupling of the methine resonances.

Variation in cPHB content as a function of strain and growth medium

Values are average of 3 determinations. MSG, minimal salts medium, 0.5% glucose, CAA, 0.02% casamino acids, CB, 2% Bacto-tryptone, 0.5% yeast extract, 10 mM KCl, 2.5 mM NaCl, CB, 10 mM CaCl2, 4.5 mM MnCl2, 100 mM KCl in 10 mM MES, pH 6.3.

| Strain | Medium  | Crotonic acid μg/mg dry wt cells | PHB μg/mg dry wt cells |
|--------|---------|---------------------------------|-----------------------|
| K12    | MSG     | 2.15                            | 4.3                   |
| K12    | SOB     | 2.54                            | 5.3                   |
| K12    | CB      | 3.12                            | 6.2                   |
| J M101 | MSG, CAA| 1.79                            | 3.6                   |
| J M101 | SOB     | 2.74                            | 5.5                   |
| J M101 | CB      | 3.25                            | 6.5                   |
| D H5α | SOB     | 2.66                            | 5.3                   |
| D H5α | CB      | 3.15                            | 6.3                   |
| C A10 | SOB     | 2.60                            | 5.2                   |
| C A10 | CB      | 3.04                            | 6.1                   |

* Values are estimates based on the assumption that the yield of crotonic acid from cPHB under the assay conditions is approximately the same as that for PHB granules used as standards (50%).

Distribution of cPHB among cell fractions of log-phase and competent E. coli J M101

Fractionation procedure and method for cPHB determination are described under "Experimental Procedure." Each value is the average of 3 determinations.

| Fraction   | Log-phase cells | Competent cells |
|------------|-----------------|-----------------|
|            | PHB μg/mg protein | PHB μg/mg protein |
| Cytosol    | 10.2            | 11.8            |
| Cytosol    | 3.8             | 3.9             |
| Ribosome   | 12.1            | 15.9            |
| Membranes  | 1.6             | 2.0             |

* Values are estimates based on the presumption that the yield of crotonic acid from cPHB under the assay conditions is the same as that for PHB granules used as standards (50%).

Fig. 5. Evidence for the presence of cPHB in the proteins of E. coli J M101. The figure shows chromatograms of crotonic acid standard (A) or crotonic acid formed when the samples were heated in concentrated sulfuric acid at 120 °C for 40 min (B–D) (see “Experimental Procedures”). Chromatography was done on an Aminex HPX-87H ion exclusion organic acid analysis column with 0.014 N sulfuric acid as eluant at a flow rate of 1 ml/min. A, crotonic acid standard, 0.2 μg; B, cold 5% trichloroacetic acid precipitate of log-phase cells of J M101 (30 μg of protein). C, sample as in B after heating in 5% trichloroacetic acid at 90 °C for 15 min. Protein was reprecipitated by cooling for 1 h on ice. D, lyophilized supernatant from sample C.

for cPHB content. As can be seen in Fig. 5C, this treatment removed >97% of cPHB from the acid-precipitable fraction. The process resulted in only slight degradation of the proteins as indicated by the appearance of polypeptide bands on SDS-polyacrylamide gel electrophoresis gels (not shown). The proteins in this residue contain very little cPHB and are referred to as cPHB-free proteins. Continuing the reaction for longer periods did not release additional amounts of cPHB and resulted in increased degradation of the proteins. The lyophilized supernatant (5% trichloroacetic acid-soluble fraction) yielded crotonic acid when heated in concentrated sulfuric acid (Fig. 5D), indicating that the trichloroacetic acid hydrolysis yielded small oligomers of PHB or the monomer, 3-hydroxybutyrate.

cPHB Proteins—Considering the insolubility of PHB in water, its concentration in the cytoplasm suggests it is sequestered within hydrophobic pockets of other macromolecules, most probably protein. To detect specific polypeptides associated with cPHB, Western blots of SDS-PAGE gels of J M101 cell fractions were probed with polyclonal anti-PHB IgG that had been preadsorbed with cPHB-free proteins (see above). The adsorbed antisera gave a strong positive reaction to synthetic PHBmon (not shown), but did not react significantly to cPHB-free proteins, with the exception of a diffuse band at approximately 12,000 Da (Fig. 6, lane 1). All cell fractions contained a number of polypeptides that reacted to the antisera, with the highest concentration of cPHB polypeptides appearing in the ribosomal fraction (lane 4). Cell fractions of competent cells had essentially the same distribution of cPHB polypeptides as log-phase cells, and no new polypeptides were observed (not shown).

When the cytoplasmic fraction was treated with proteinase K (200 μg/ml) for 2 h at 37 °C before SDS-gel electrophoresis and Western blotting, no polypeptides or cPHB-containing bands were detected.
were observed at \( M_r > 16,000 \); however, there was a diffuse band of degraded proteinaceous material in the molecular weight region of \(-2,000–15,000 \) which reacted positively to the adsorbed cPHB antisera (not shown). It is possible that the actual \( M_r \) range of these presumed cPHB-peptides or polypeptides is less, since the uncharged polyester may have a conspicuous influence on the electrophoretic mobility of small molecules. cPHB was not released by this enzymatic proteolysis; less than 1% of the polyester in the hydrolysate was chloroform-extractable (\(-10\%\)). Most if not all of this chloroform-soluble fraction of cPHB, until recently believed to be the only cPHB in \( E. \) coli, forms complexes with calcium polyphosphate in the plasma membranes (Reusch and Sadoff, 1988) that function as voltage-activated calcium channels (Reusch et al., 1995). We surmise that most of the remainder of cPHB formed on induction of competence is incorporated into existing PHB proteins, since no new polypeptides were observed and earlier studies showed that there is no de novo synthesis of protein during the development of competence (Reusch et al., 1986). In this study, we have shown that the great majority of cPHB in both log-phase and competent cells is chloroform-insoluble and associated with proteins.

There are a number of ways in which cPHB may associate with protein. As an amphiphilic molecule, cPHB can form hydrophobic bonds via its methyl groups or act as a hydrogen-bond acceptor or form coordinate bonds to bridging cations via its carboxy or hydroxy group. Individually, such noncovalent bonds are weak, but the frequency at which the methyl groups and ester carboxyl oxygens repeat along the PHB backbone allows each polymer molecule to have many such interactions. The number and strength of these bonds would be determined by the primary structure and three-dimensional geometry of the protein at the binding site(s). The tenacity of the bonding, as evidenced by its ability to withstand heating in sodium lauryl sulfate, extraction with hot chloroform, and proteolysis, suggests that there is also a covalent bond between the protein and the terminal carboxy or hydroxy group of cPHB. Thus far, we have succeeded in removing PHB from its complexes with proteins only by treatments which effect hydrolysis of the ester bonds, i.e. heating in dilute acid or methanol:chloroform mixtures.

PHB is a linear, flexible, polymer chain, with a high density of “sticky” sites, alternating polar and nonpolar. This amphiphilic nature may enable cPHB to adjust or reverse the polarity of specific regions of the protein. When the methyl groups of cPHB are bound to nonpolar residues, the ester carboxyl oxygens form a hydrophilic surface which may associate with polar molecules. Conversely, when the carboxyl oxygen bond to polar amino acids or their salts, the hydrophobic coat of cPHB can attract hydrophobic molecules or promote insertion of the peptide into a bilayer.

In summary, we find that \( E. \) coli cells contain significant amounts of cPHB. In log-phase cells, most cPHB is associated with specific proteins in both the cytoplasm and membranes, but particularly with proteins of the ribosomal fraction. It seems probable that the cPHB is covalently bound and that the cPHB proteins constitute yet another class of protein conjugates. When cells are made genetically competent, there is de novo synthesis of cPHB; about 10% of the newly synthesized polyester forms complexes with calcium polyphosphate in the plasma membranes (Huang and Reusch, 1995; Reusch and Sadoff, 1988). Our studies show that cPHB is widely distributed within the cell, wherein it is largely associated with proteins. Surprisingly, the majority of this lipophilic polyester is associated with proteins in the cell cytoplasm, particularly proteins of the ribosomal fraction (Fig. 6 and Table IV). Studies are now in progress to identify the proteins associated with cPHB.

When cells are made genetically competent, there is de novo synthesis of cPHB amounting to 15–20% of the extant cPHB (Fig. 1B and Table III (Huang and Reusch, 1995; Reusch and Sadoff, 1988). Only a portion of the newly synthesized cPHB is chloroform-extractable (\(-10\%\)). Most if not all of this chloroform-soluble fraction of cPHB, until recently believed to be the only cPHB in \( E. \) coli, forms complexes with calcium polyphosphate in the plasma membranes (Reusch and Sadoff, 1988) that function as voltage-activated calcium channels (Reusch et al., 1995). We surmise that most of the remainder of cPHB formed on induction of competence is incorporated into existing PHB proteins, since no new polypeptides were observed and earlier studies showed that there is no de novo synthesis of protein during the development of competence (Reusch et al., 1986). In this study, we have shown that the great majority of cPHB in both log-phase and competent cells is chloroform-insoluble and associated with proteins.

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Sadoff, 1988; Reusch et al., 1995; Castuma et al., 1995), and the remainder is incorporated into some extant cPHB proteins. These findings imply that PHB is a fundamental constituent of biological cells with important physiological roles in addition to its function in ion transport. Considering the solvent and salt-complexing properties of this polyester, we suggest it is involved in the inter- and intracellular transport of macromolecules.

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