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Isolation of Intact Chains of Polyphosphate from "*Propionibacterium shermanii*" Grown on Glucose or Lactate

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A procedure is presented for the isolation of intact polyphosphate (poly P) from "*Propionibacterium shermanii*." It is demonstrated, by including [^{32}P]poly P during the extraction, that this procedure does not hydrolyze the poly P, and it is shown that two other widely used procedures do cause breakdown of the poly P. The procedure presented allows isolation of three fractions, short-chain poly P which is soluble in trichloroacetic acid, long-chain poly P which is soluble at neutral pH, and long-chain poly P which is present in volutin granules. Cells which had been grown on lactate did not contain short-chain poly P but did contain a high amount of long-chain poly P, which accumulated to 3% of the cell dry weight. At least 70% of this poly P was present in volutin granules. The poly P ranged in length from 250 to 725 phosphate residues and was the same average size as that synthesized in vitro by the poly P kinase from "*P. shermanii*." This indicates that the poly P kinase is responsible for catalyzing the synthesis of the poly P. In contrast to cells grown on lactate, those which had been grown on glucose did not contain volutin granules, did contain short-chain poly P and had 100-fold less long-chain poly P than lactate-grown cells. We propose that during the fermentation of glucose, the amount of poly P is lower than during growth on lactate because it is continuously utilized as a substrate in the phosphorylation of glucose.

Polyphosphates (poly P), polymers of phosphate linked by phosphoanhydride bonds, have been detected in almost all organisms studied including bacteria, yeasts, fungi, plants, and animals (13). From the considerable amount of research which has been conducted in the past 30 years, several roles have been proposed for poly P in metabolism (13, 14). A major obstacle in demonstrating any function for poly P is the lack of adequate methods, including those for isolating and determining the sizes of poly P. The chain lengths of poly P, which may range from 3 to greater than 1,000 phosphate residues, vary among different organisms and usually vary during the growth of any particular organism (13). The importance of studying the size of poly P was indicated in 1958 when Langen and Liss (16) found that, in yeast cells, $^{32}P_i$ was incorporated first into pools of long-chain poly P and later into pools of shorter-chain poly P.

Poly P molecules are labile, and hydrolysis is catalyzed by cations, alkali, and acid (17), yet the degree of degradation which may occur during extraction has never been accurately determined. We present here a new procedure which extracts poly P from "*Propionibacterium shermanii*," including that of volutin granules, and demonstrate that this procedure does not hydrolyze the poly P to shorter chains. To examine the chain lengths, we electrophoresed the poly P on polyacrylamide gels.

The only poly P-dependent enzymes present in the main fermentation path of "*P. shermanii*" are poly P kinase, which catalyzes the synthesis of exclusively long-chain poly P from ATP (reaction 1), and poly P glucokinase, which catalyzes the transfer of the terminal phosphate group from poly P to glucose forming glucose-6-phosphate (reaction 2) (32). The mechanisms of catalysis of these reactions have been studied in detail (18, 22, 23; C. A. Pepin, N. A. Robinson, and H. G. Wood, Fed. Proc. 45:1610; N. A. Robinson and H. G. Wood, Fed. Proc. 45:1610).

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$$\text{poly } P_n + \text{ATP} \rightleftharpoons \text{poly } P_{n+1} + \text{ADP} \quad \text{(reaction 1)}$$

$$\text{glucose} + \text{poly } P_n \rightarrow \text{glucose-6-phosphate} + \text{poly } P_{n-1} \quad \text{(reaction 2)}$$

In continuing our efforts to determine the role of poly P in the metabolism of "*P. shermanii*," we investigated the poly P from cells grown on lactate or on glucose. Our results support the conclusion that the poly P kinase is responsible for the synthesis of poly P and that poly P is utilized via poly P glucokinase during fermentation of glucose.

MATERIALS AND METHODS

Materials. Before use, phenol was distilled, added to an equal volume of chloroform, and saturated with 0.1 M ammonium acetate (pH 6.5); hereafter this is referred to as phenol-chloroform. Acrylamide (twice crystallized) was from Accurate Chemical and Scientific Corp. (Westbury, N.Y.); *N*,*N*'-methylene-bisacrylamide was from Bio-Rad Laboratories (Richmond, Calif.). Dowex-50W, toluidine blue O, and type 65 and type 35 poly P were from Sigma Chemical Co. (St. Louis, Mo.). Electrophoresis grade agarose was from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.); urea was from BDH Chemicals (Poole, England). Norit A decolorizing carbon was from Fisher Scientific Co. (Pittsburgh, Pa.). Poly P glucokinase, purified 1,000-fold to a specific activity of 15 U/mg (18), and poly P kinase, purified to 50% homogeneity to a specific activity of 0.5 U/mg (22, 23; N. A. Robinson and H. G. Wood, Fed. Proc. 45:1610), were generously provided by C. A. Pepin and N. A. Robinson, respectively.

Cell growth and harvest. "*P. shermanii*" ST33 was grown in either 3% lactate or 1.5% glucose as described by Wood and Goss (32) except that the glucose medium contained 5 g of tryptone and, as the buffer, 0.4 g of KH_2PO_4 and 4 g of K_2HPO_4 per liter. For both carbon sources, the metal content was changed to contain (per liter of medium): $MgCl_2 \cdot 6H_2O$, 40.7 mg; $MnCl_2$, 5.9 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $CaCl_2$, 14 mg; $ZnCl_2$, 0.82 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1

mg; SeO_2, 0.1 mg. Cultures were stored at 5°C as agar stabs on the appropriate carbon substrate, transferred to liquid medium, and grown at 30°C. Cell growth was monitored by measuring the A_{660}, and dry weight was determined from a standard curve of optical density at 660 nm versus constant dry weight (8).

For growth studies, duplicate samples which ranged in volume from 50 to 300 ml depending on the point in growth were removed at various times. To inactivate enzymes which might hydrolyze the long-chain poly P during the cell harvest, one sample was poured into an ice-cold solution of trichloroacetic acid (TCA)-acetone so that the final concentrations were 2% (wt/vol) TCA and 67% (vol/vol) acetone, respectively. This treatment extracted acid-soluble molecules, such as short-chain poly P, into the harvest medium. However, owing to the high amount of background P_i in the growth medium, it was not possible to assay for this short-chain poly P. The second sample of cells was not treated with TCA-acetone and was used for determination of the amount of short-chain poly P extracted by step 1 below. The cells were collected by centrifugation at 5°C and 5,000 × *g* for 15 min and stored at −80°C.

Extraction and purification of poly P. All work was at 23°C unless otherwise indicated, and all centrifugations were carried out in 1.5-ml tubes at 15,000 × *g* with an Eppendorf microcentrifuge. A 100% recovery was obtained when [^{32}P]poly P was included in the procedure, except at step 5 and after treatment with decolorizing carbon (after step 1), in which recoveries ranged from 80 to 100%.

(i) Step 1. Acid-soluble poly P was extracted by initially suspending cells (approximately 0.1 g [wet weight]) in 0.3 ml of ice-cold 2% (wt/vol) TCA. The suspension was centrifuged, and the residue was suspended in a 1-ml solution of ice-cold TCA-acetone (0.7%:67%). After centrifugation, the cell residue was washed with ice-cold 67% (vol/vol) acetone. The TCA-containing solutions were combined, and acetone was removed by extraction with an equal volume of phenol-chloroform (1:1, vol/vol). The poly P of this fraction is designated short-chain poly P.

(ii) Step 2. The portion of the long-chain poly P which is soluble at neutral pH was extracted from lactate-grown cells by suspension of the cell residue from step 1 in 0.5 ml EDTA (2 mM) and titration of the suspension to pH 7 to 8 with 0.2 M LiOH. The final Li^+ concentration was about 30 mM. The suspension was centrifuged for 5 min, and the supernatant containing the extracted poly P was purified further as described in step 4. The poly P of this fraction is designated long-chain soluble poly P, although for cells producing a variety of sizes, all sizes which are soluble and not extractable with TCA would be obtained.

(iii) Step 3. The cell residue of step 2 was suspended in EDTA and titrated as in step 2. Approximately 0.2 ml of the phenol-chloroform (1:1, vol/vol) solution was added, and then the suspension was shaken vigorously and centrifuged for 10 min. The poly P in the aqueous layer was purified further as described in steps 4 and 5. The poly P of this fraction is designated long-chain granule poly P.

(iv) Step 4. Protein was removed separately from the poly P solutions of steps 2 and 3 by extraction with an equal volume of phenol-chloroform (1:1, vol/vol) followed by three successive extractions with chloroform.

(v) Step 5. After step 4, the long-chain poly P from lactate-grown cells was separated from nucleic acids by precipitation of the poly P at neutral pH as the Mg^{2+} salt. To accomplish this, $MgCl_2$ was added to give a final ratio of 2 mol of Mg^{2+} per mol of phosphate in the poly P. The Mg^{2+}-poly P solution was placed in an ice-water bath for 15 min and then centrifuged for 3 min. The supernatant contained nucleic acids and was discarded. The poly P precipitate was suspended in water and solubilized by the addition of a few beads of Dowex-50W (H^+ form). The poly P solution was brought to pH 7 by the addition of either LiOH or 1 M Tris hydrochloride (pH 7.6), and then EDTA was added to a concentration equal to the poly P concentration to ensure complete chelation of Mg^{2+}. Precipitation with $MgCl_2$ requires a concentration of at least 0.15 mg of poly P per ml. At pH 7, only chain lengths greater than 100 are precipitated; shorter chains precipitate at higher pH values, but at the high pH, the poly P is hydrolyzed partially.

Two other extraction procedures were compared with the method described above. Lactate-grown cells from the log phase of growth were treated successively with acid, salt, and alkali, the latter at pH 9 and then at pH 12 as described by Langen and Liss (16), or with sodium hypochlorite as described by Harold (4). For the latter procedure, 5 mM EDTA and 1 mM NaF were included during the extraction as described by Rao et al. (20). The sodium hypochlorite was either prepared from bleaching powder (calcium hypochlorite) as described by Williamson and Wilkinson (31) or Chlorox liquid bleach was used directly. After both procedures, protein was extracted before electrophoresis as described above in step 4.

Assays for poly P. Three different methods, A, B, and C, were used to determine the amount of poly P extracted from cells.

Method A. Acid-soluble poly P (short chain), extracted during step 1 of the procedure, was treated with Norit A decolorizing carbon to remove nucleotides. After this treatment, a portion of the extract was boiled in 1 N HCl for 10 min, and then the acid-hydrolyzable phosphate was determined by the method of Josse (11).

Method B. The poly P content in the extracts of lactate-grown cells from steps 2 to 5 was routinely determined by measuring the metachromatic reaction of toluidine blue at 630 nm (3). The assay was performed by adding less than 10 μl of the poly P sample to tubes containing 0.75 ml each of acetic acid (0.2 N) and toluidine blue (30 mg/liter). The amount of poly P was determined within 15 min by comparison with a standard curve produced by using 1 to 5 μg of type 65 poly P. The amount of poly P extracted (steps 2 and 3) remained unchanged through the purification (steps 4 and 5), and results with the toluidine blue assay were identical (±5%) with results obtained by the determination of acid-hydrolyzable phosphate for a sample of poly P which had been purified through step 5. There is not a dependency of metachromasy on chain length for poly P which is longer than at least 30 residues (29). In addition, the amount of poly P in lactate-grown cells was high enough that compounds such as nucleic acids, which have been reported to interfere with the toluidine blue assay (3, 30), did not alter results. The amount of poly P estimated by the toluidine blue assay was the same before and after the removal of nucleic acids at step 5.

Method C. The fraction of poly P of glucose-grown cells which was not extracted with TCA (step 1) was present in very low amounts and was extracted by step 3 of the procedure (step 2 was omitted). The poly P was identified, and the amount was determined spectrophotometrically by reaction with poly P glucokinase coupled to glucose-6-phosphate dehydrogenase (18). The assay contained (in a final volume of 0.4 ml): 100 mM Tris hydrochloride (pH 7.6), 6.6 mM glucose, 6.6 mM $MgCl_2$, 0.6 mM NADP, the sample

from the extraction procedure, glucose-6-phosphate dehydrogenase (0.9 U), and poly P glucokinase (0.04 U). The former enzyme was added, and the absorbance was monitored to be sure no glucose-6-phosphate was present. The reaction was then initiated with poly P glucokinase, and the absorbance was monitored continuously with a Gilford 250 spectrophotometer until the reaction terminated. Then a known amount of poly P was added to ensure there was no inhibition of the reaction. In a separate reaction, the sample containing poly P was omitted, and the absorbance change which occurred upon addition of poly P glucokinase was measured and subtracted from the absorbance change measured in the presence of the poly P sample. The concentration of NADP was at least sixfold higher than that of the glucose-6-phosphate produced by the poly P glucokinase, allowing quantitative conversion of poly P to 6-phosphogluconate (7). Cuvettes were soaked in chromic acid cleaning solution to remove residual poly P from previous experiments.

Analysis of poly P by gel electrophoresis. Gel electrophoresis was performed with either a Tris borate buffer (24) for 15% polyacrylamide gels (0.03 by 16 by 40 cm) or a Tris acetate buffer (24) for 2% polyacrylamide–0.75% agarose gels (0.18 by 16 by 40 cm). For the 15% gel, chain lengths of poly P less than 100 residues were determined by counting bands on the gel as previously described (22, 23). For the 2% gel, chain lengths were determined by comparison with the migration of standards which were prepared and sized by a recently developed procedure (J. E. Clark and H. G. Wood, Anal. Biochem., in press). When necessary, poly P was concentrated by precipitation with acetone. Tris hydrochloride (1 M, pH 7.6) was added to a concentration of 0.1 to 0.2 M, and then acetone (2 volumes) was added. The mixture was frozen on dry ice and incubated at −25°C for 15 min, and then the precipitated poly P was recovered after centrifugation for 5 min.

Identification and purity of poly P extracted from lactate-grown cells was determined by reacting the poly P with poly P glucokinase and then analyzing the shortened poly P product by gel electrophoresis (18, 19). The reaction mixture contained (in a final volume of 0.8 ml): 100 mM Tris hydrochloride (pH 7.6), 6.6 mM glucose, 6.6 mM $MgCl_2$, and 50 μg of poly P. The reaction was initiated by adding 0.04 U (micromoles of glucose-6-phosphate produced per minute) of poly P glucokinase and terminated by the addition of EDTA (12 mM, final concentration) and an equal volume of phenol-chloroform (1:1, vol/vol). The mixture was shaken vigorously and then centrifuged for 7 min. The aqueous layer containing the poly P was concentrated as described above and then electrophoresed.

To demonstrate that the extraction procedure does not hydrolyze poly P, we included ^{32}P-labeled poly P after step 1 during the isolation of poly P from lactate-grown cells. The [^{32}P]poly P was synthesized in vitro by reaction of the poly P kinase from "*P. shermanii*" with [γ-^{32}P]ATP in the presence of histone (22). The sizes of the [^{32}P]poly P and of the [^{32}P]poly P which had been subjected to the cellular extraction were analyzed by electrophoresis on a 2% polyacrylamide gel. The gel was dried and exposed to X-ray film at −25°C overnight.

The presence of contaminant proteins was ruled out by staining gels by two different methods (21, 26). Contaminant RNA was identified by its hydrolysis to shorter chain lengths when treated with RNase or when boiled for 2 min in 0.2 N NaOH (25). The RNA before and after hydrolysis was electrophoresed on a 15% polyacrylamide gel, and the gel was stained with toluidine blue. RNA stains bluish purple, while poly P stains bright pink.

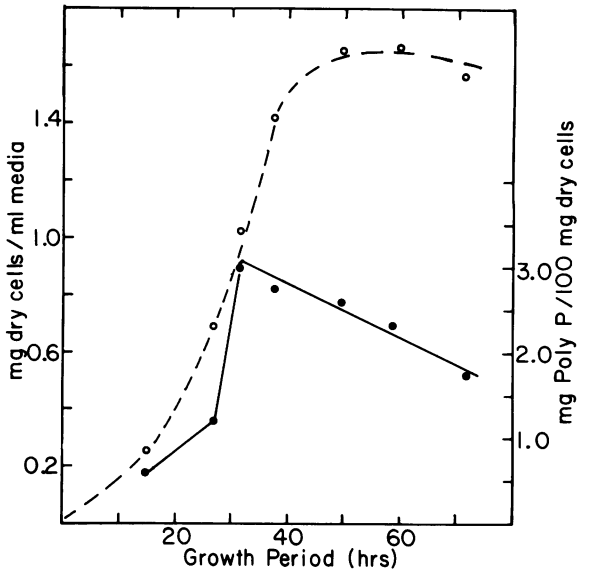

FIG. 1. Amount of long-chain poly P in "*P. shermanii*" grown on lactate. The dashed line represents cell growth, and the solid line represents the amount of soluble and granule poly P, determined by assay method B with toluidine blue. Data points for the amount of poly P are an average of at least two determinations which were within 10% of each other. For one set of determinations, cells were harvested in the presence of TCA-acetone as described in Materials and Methods. These cells did not contain short-chain poly P.

Electron microscopic studies. "*P. shermanii*" was grown to the log phase in 2% lactate, harvested by centrifugation, and used immediately for electron microscopic studies. A sample of the fresh cells was fixed. The remaining cells were treated as described in steps 1 to 3 of the extraction procedure, with a sample fixed after each step essentially as described by Held (6). The cell samples were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, and then, after dehydration, stained with 1% uranyl nitrate in 100% ethanol. The samples were embedded in Spurr medium, and then thin sections (70 nm) were prepared and stained with uranyl acetate and lead citrate. The electron microscope was a JEOL 100C.

RESULTS

Amount of poly P during growth in lactate or glucose. Poly P which are extractable with 2% TCA are commonly referred to as acid soluble and have been found, in a variety of organisms, to consist of chain lengths of less than 20 residues; we refer to this acid-soluble poly P as short-chain poly P. The poly P which is not extracted with 2% TCA consists of longer chains, and we refer to this acid-insoluble poly P as long-chain poly P.

"*P. shermanii*" grown on lactate accumulated a high amount of poly P, producing a maximum of 3% of the cell dry weight during the mid-log phase of growth (Fig. 1). Throughout growth, the poly P was found to be exclusively long chain, and there was no short-chain poly P. This is in accord with results reported by Kulaev (13). From electron microscopic studies (below), the majority of the poly P apparently was present in volutin granules.

In contrast to growth on lactate, cells grown on glucose did not contain volutin granules (data not shown) and had a

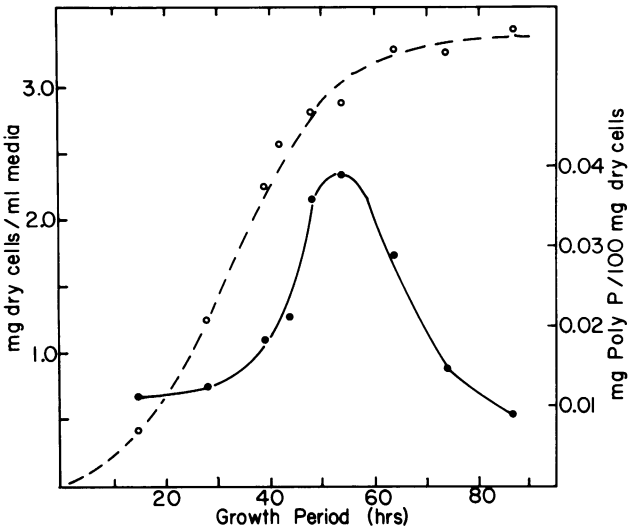

FIG. 2. Amount of long-chain poly P in "*P. shermanii*" grown on glucose. The dashed line represents cell growth, and the solid line represents the amount of poly P extracted at step 3 and determined by assay method C with poly P glucokinase. These cells did not contain granule poly P. Data points for the amount of poly P are an average of at least two determinations which were within 15% of each other. For one set of determinations, cells were harvested in the presence of TCA-acetone as described in Materials and Methods. The amount of short-chain poly P extracted during step 1 is not shown.

very low level of long-chain poly P (Fig. 2). This fraction of poly P was quantitated by a spectrophotometric assay with poly P glucokinase (assay method C). The highest amount was found in cells from the late log phase and was only 0.04% of the cell dry weight. Cells grown on glucose also contained short-chain poly P which was extracted with TCA (step 1). There was at least twice as much of this poly P throughout growth (data not shown) as there was long-chain poly P. We determined the amount of the short-chain poly P by measuring the P_i produced upon hydrolysis in boiling acid (assay method A). While this is the typical assay used for the determination of poly P, it does not prove that the acid-hydrolyzable phosphate was derived from poly P. We were unable to obtain sufficiently pure material to characterize further either of the fractions of poly P from glucose-grown cells.

Extraction procedure and examination of lactate-grown cells by electron microscopy. We studied the effect of our extraction procedure on the volutin granules present in "*P. shermanii*" during growth on lactate (Fig. 3A). Step 1 of the extraction procedure, involving treatment with a solution of TCA-acetone, appeared to break up the large granules shown in Fig. 3A into smaller granules as shown in Fig. 3B. This first step was performed to permeabilize cells and to extract any acid-soluble poly P; however, no poly P was obtained from lactate-grown cells in this fraction. Step 2, involving treatment of the cell residue at low ionic strength, extracted only 30% of the poly P present in these cells. The optimum pH for extraction was found to be between 7 and 8. Examination of the granules after step 2 by electron microscopy (not shown) revealed no obvious change in either the size or number of poly P granules. Repeated extractions did not release any additional poly P, nor was there an increase in the amount of poly P obtained by increasing the incubation time for the extraction. Thus, this fraction of poly P, which was extracted by a mild procedure, was not tightly complexed within the cells. The poly P obtained at step 2 may not have been associated with the granules and, therefore, is designated the soluble fraction of poly P. Step 3 of the procedure, which includes treatment with a solution of phenol-chloroform, extracted the remaining 70% of the poly P. In addition, this procedure also extracted the volutin granules (Fig. 3C). Since the volutin granules disappeared, this fraction of poly P is designated the granule fraction.

We compared the size of the soluble and granule fractions by gel electrophoresis (data not shown) and found that these fractions of poly P were identical in size. If step 2 was omitted from the procedure, all the poly P of the soluble and granule fractions was extracted in step 3. Overall, at least

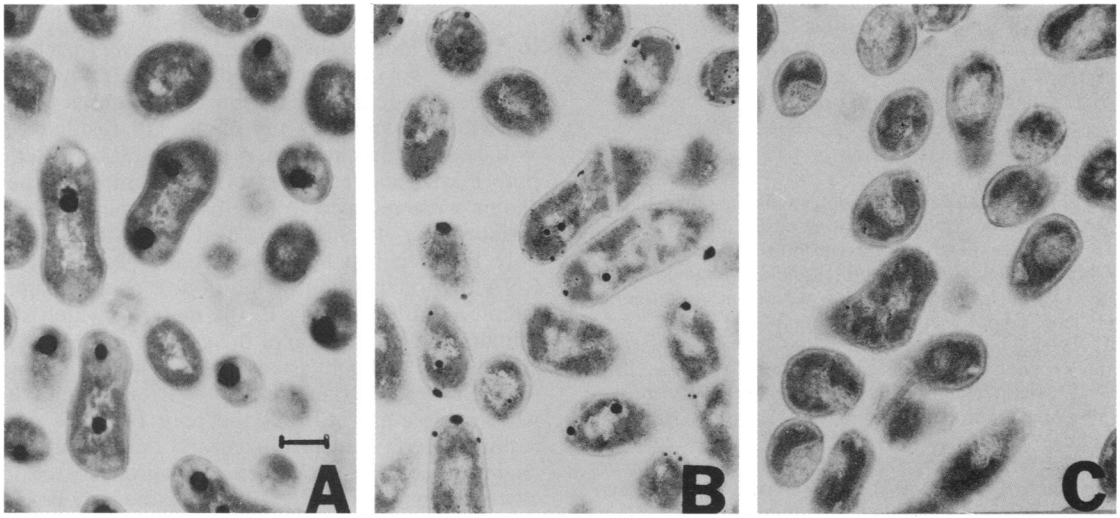

FIG. 3. Effect of extraction procedure on poly P-containing granules of lactate-grown cells. "*P. shermanii*" was grown on 2% lactate and harvested in the late log phase. The extraction procedure as described in Materials and Methods was performed on the cells with samples removed as indicated in the text and immediately fixed for electron microscopic studies. (A) Fresh cells containing large electron-dense volutin granules; (B) cells of panel A after treatment with TCA-acetone, step 1 of the extraction procedure; (C) cells of panel A after steps 1 to 3 of the extraction procedure. The bar shown in panel A represents 360 nm.

95% of the total poly P was extracted as estimated by the amount of acid-hydrolyzable phosphate remaining in the cell residue.

Purity of poly P determined by gel electrophoresis. To identify the material extracted from lactate-grown cells as poly P, as well as to determine purity, it was reacted with poly P glucokinase for various lengths of time, and the products were analyzed by polyacrylamide gel electrophoresis. Any material which stained with toluidine blue on the gel but which was not utilized by the poly P glucokinase was considered to be a contaminant. The poly P obtained at step 3 of the extraction procedure contained RNA (Fig. 4, lanes 3 to 5). The long-chain poly P, which was produced by the lactate-grown cells, migrated only partially into the 15% polyacrylamide gel. Migration on this gel is linear with the logarithm of lengths from 15 to 110 residues (Clark and Wood, Anal. Biochem., in press), and it is evident when the poly P was reacted with poly P glucokinase that the long chains were reduced to lengths of somewhat less than 100 residues (Fig. 4, lanes 4 and 5). Lanes 7 to 11 of Fig. 4 are of poly P which had been separated from the contaminating RNA by precipitation of the poly P as the magnesium salt (step 5). With this poly P, the material which stained with toluidine blue at the top of the gel was utilized by the poly P glucokinase and converted to short chains of somewhat less than 100 residues (lanes 8 and 9). After residual long chains were completely utilized, the poly P chains of about 100 residues were in turn utilized and converted to very short chains (lane 10), a characteristic property of the poly P glucokinase reaction (18, 19). Thus, the poly P was shown to be free of RNA, and it was also found to be free of protein. It was necessary to obtain pure poly P to determine its size by gel electrophoresis (see below).

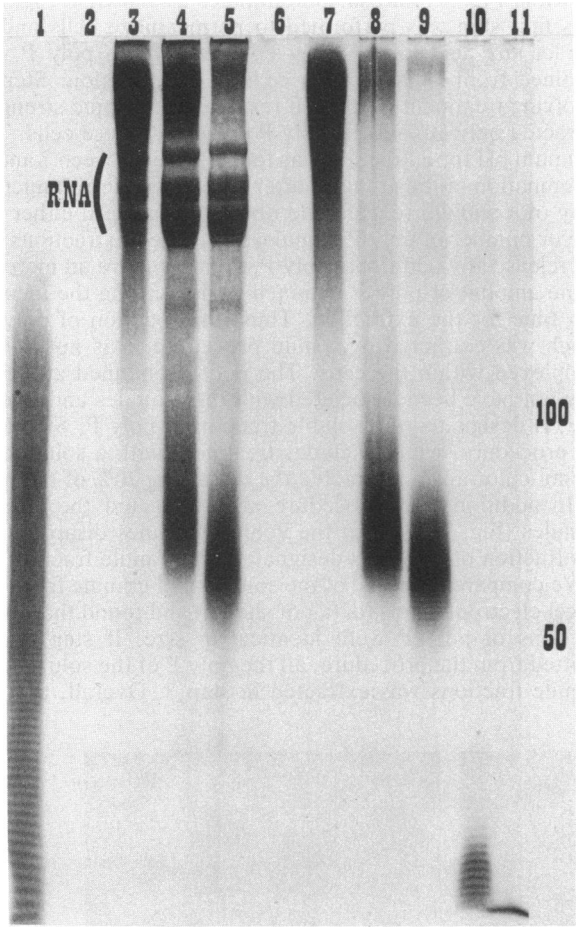

FIG. 4. Use of poly P glucokinase to determine the purity of poly P extracted from "*P. shermanii*" grown on lactate. The 15% polyacrylamide gel was electrophoresed until the marker dye, bromophenol blue, had migrated 16 cm. Lane 1 contained Sigma type 35 poly P (15 μg). Lanes 3, 4, and 5 were from an extract of steps 3 and 4; lane 3 contained 10 μg of poly P. Lanes 4 and 5 contained poly P which had been reacted with poly P glucokinase for 10 and 10.5 min, respectively, before electrophoresis. Lanes 7 to 11 contained poly P extracted during step 3 and then purified further through step 5 by precipitation as the Mg^{2+} salt. Lane 7 contained 10 μg of poly P. The poly P in lanes 8, 9, and 10 had been reacted with poly P glucokinase for 10, 10.5, and 11 min, respectively, before electrophoresis. The poly P in lane 11 had been reacted with poly P glucokinase for 11.5 min, but the migration of the poly P was retarded owing to the presence of high salt (from glucose-6-phosphate). The location of contaminant RNA is indicated on the left. The chain lengths of poly P (in number of residues; indicated on the right) were determined by counting bands on the gel starting with poly P_4 (not shown) as described previously (19, 22, 23).

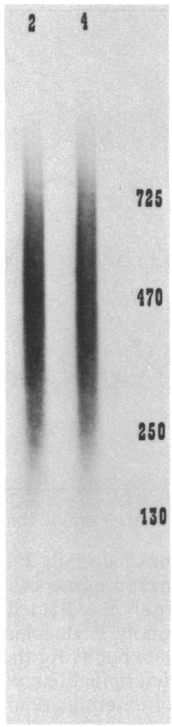

FIG. 5. Demonstration that poly P was not hydrolyzed during the extraction procedure. [^{32}P]poly P was synthesized with poly P kinase and then was included during the extraction of long-chain poly P from lactate-grown "*P. shermanii*." Electrophoresis was on a 2% polyacrylamide–0.75% agarose gel until bromophenol blue had migrated 14 cm. The gel was dried and then exposed to X-ray film overnight at −25°C. Lane 2 contained [^{32}P]poly P (30,000 cpm) before inclusion in the extraction procedure. Lane 4 contained [^{32}P]poly P (30,000 cpm) which was included in the extraction procedure after step 1 and through step 5. Chain lengths (number of residues) of poly P are indicated to the right of the gel and were determined by comparison with the migration of poly P standards (Clark and Wood, Anal. Biochem., in press) such as those shown in Fig. 6.

Size of poly P from lactate-grown cells and proof the poly P was not hydrolyzed during extraction. To demonstrate that

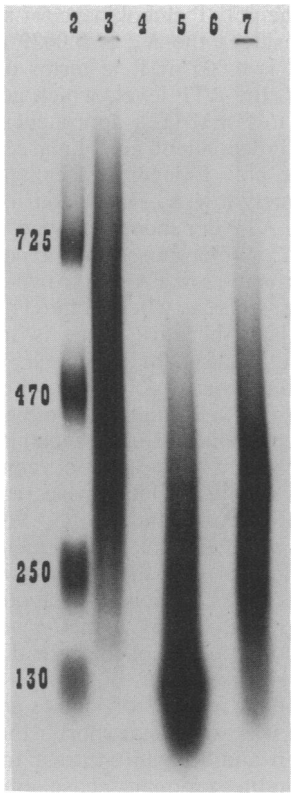

FIG. 6. Size of poly P extracted by our procedure from lactate-grown "*P. shermanii*" and comparison with sizes obtained by other extraction procedures. The poly P was electrophoresed on a 2% polyacrylamide–0.75% agarose gel until the bromophenol blue had migrated 14 cm and then was stained with toluidine blue. The lanes contained the following: lane 2, poly P standards (Clark and Wood, Anal. Biochem., in press) having chain lengths (in residues) as indicated to the left; lane 3, poly P (20 μg) isolated from the combined steps 2 and 3 and purified through step 5 by the procedure presented in this paper; lane 5, the combined poly P (20 μg) obtained after extraction with salt and alkali by the procedure of Langen and Liss (16); lane 7, the combined poly P (20 μg) extracted by the procedure of Harold (4).

the poly P isolated from cells was of native size and not hydrolyzed by the extraction procedure reported here, we performed the procedure with lactate-grown cells in the presence of ^{32}P-labeled poly P. The majority of the radioactive poly P, which was synthesized with poly P kinase (22, 23), ranged in length from 250 to 725 residues (Fig. 5, lane 2). There was no hydrolysis of the [^{32}P]poly P (lane 4) to shorter chain lengths during the extraction and purification of the poly P. With the low-percentage (2%) polyacrylamide gel of Fig. 5, the migration of poly P is linear with the logarithm of lengths of poly P from about 400 to 800 residues (Clark and Wood, Anal. Biochem., in press). Significant hydrolysis of the [^{32}P]poly P would have been detected as a decrease in the amount of longer-chain poly P (around 700 residues) and an increase in the amount of shorter chain lengths.

In addition to studies of the extraction procedure, we also investigated the possibility that the cells could have catalyzed a shortening of the poly P during the cell harvest. Ice-cold TCA-acetone was added to one sample of cells immediately before harvest, and another sample was harvested without treatment. For cells in the early and mid-log phases, this treatment had no effect on the size of the poly P. It was the same size as that synthesized in vitro by the poly P kinase (compare lane 2 of Fig. 5 with lane 3 of Fig. 6); the majority ranged in length from about 250 to 725 residues, with an average length of about 500 residues. In contrast, cells from later in growth did shorten the poly P during the cell harvest. Including TCA-acetone eliminated this shortening in cells in the late log phase, and the poly P was the same size as shown in lane 3 of Fig. 6. However, for cells in the stationary phase, even the TCA-acetone-treated cells had somewhat shorter poly P. There was less poly P of 700 residues and more poly P of 200 residues, with an estimated average size of about 400 residues. Thus, for cells in the late log and stationary phases, it was necessary to treat them with TCA-acetone to obtain poly P of native size, and even with this treatment, the poly P from stationary-phase cells was shorter than that from log-phase cells.

Comparison of procedures for extraction of poly P. Two widely used procedures were studied to determine whether the poly P was hydrolyzed during the extraction. With the procedure of Langen and Liss (16), we obtained results similar to those which have been reported by Kulaev (13). Only 30% of the total poly P was extracted from lactate-grown cells, with the majority extracted with the alkali treatments, and we assumed that this was the same soluble fraction which we extracted during step 2 of our procedure. However, the poly P extracted by the Langen and Liss method was much shorter in size (Fig. 6, lane 5) than that extracted by our procedure (lane 3). Most of the poly P isolated by the method of Langen and Liss consisted of chain lengths ranging from less than 100 to 350 residues, averaging about 250 residues. The majority of degradation of the poly P was apparently due to the high-salt treatment; when this step was omitted, the poly P obtained in the subsequent alkali treatments was less degraded. Kulaev (13) extracted the remaining 70% of the poly P as inorganic P_i after boiling cells in perchloric acid. Following the Langen and Liss procedure, we extracted the remaining 70% fraction by step 3 of our procedure and found that the poly P had not been hydrolyzed (data not shown). Thus, the granule fraction of poly P, which was not extracted by the Langen and Liss procedure, apparently was protected against hydrolysis.

We also examined the poly P extracted after initially treating cells with sodium hypochlorite as described by Harold (4). With Chlorox liquid bleach, about 15% of the poly P was obtained in the 1 M salt wash and contained chain lengths averaging approximately 130 residues. The remaining poly P, including that from granules, was obtained in the subsequent water extraction. The majority of the poly P of the combined fractions (Fig. 6, lane 7) contained chain lengths ranging from about 150 to 450 residues, with an average of 300 residues. When sodium hypochlorite prepared from bleaching powder (31) was used instead of Chlorox, the poly P was severely degraded and contained an entire range of sizes, present in approximately equal concentrations, from about 10 to 400 residues (data not shown).

DISCUSSION

During studies on the role of poly P in "*P. shermanii*," we found that currently available procedures for extracting poly P caused hydrolysis of the poly P. It was necessary, therefore, to develop a new isolation procedure to study the metabolism of native-size poly P. With the procedure presented, three fractions of poly P were obtained: (i) the extremely short-chain poly P, which is commonly extracted with TCA and referred to as acid-soluble poly P; (ii) the

long-chain poly P which we refer to as soluble, which is extracted at low ionic strength and neutral pH; and (iii) the long-chain granule poly P, which is present within the volutin granules of lactate-grown cells and extracted by the addition of phenol-chloroform. We demonstrated that the procedure does not cause hydrolysis to the poly P, which, to our knowledge, is the first time any procedure has been analyzed for the amount of degradation which occurs. The poly P obtained by the procedures of Langen and Liss (16) and of Harold (4) was, on the average, nearly twofold shorter than that extracted by our procedure. As would be expected from random hydrolysis, the majority of hydrolysis occurred as internal chain breakage; the recovery of poly P was too high for the loss of chain length to have been due totally to hydrolysis of terminal residues to P_i.

By isolating poly P of native size, we determined that the soluble and the granule fractions of poly P from "*P. shermanii*" grown on lactate are the same size. The ratio of granule to soluble poly P remains fairly constant at about 2 throughout growth, suggesting either that the fractions are metabolized simultaneously or that there is at least an equilibrium between the two fractions. It is possible that the soluble poly P is part of the volutin granules but less tightly complexed than the granule poly P. Other molecules which may be associated with the poly P in the propionibacteria are not known; poly P from other organisms are reportedly complexed with divalent cations (1, 10), basic amino acids (2), and proteins (9).

The poly P isolated from log-phase cells grown on lactate was the same size as that produced in vitro by the poly P kinase (reaction 1), with the majority ranging in length from about 250 to 725 residues. Although there have been reports of other enzymes which synthesize poly P (13, 14), the poly P kinase, which is present at about the same level (0.7 μmol/min per g of cells) (N. A. Robinson and H. G. Wood, Fed. Proc. **45**:1610) throughout growth (32), is the only enzyme thus far discovered in "*P. shermanii*" which could have such a role (32). The mechanism of the poly P kinase has recently been determined to be strictly processive (23; N. A. Robinson and H. G. Wood, Fed. Proc. **45**:1610) such that the enzyme synthesizes (in the forward reaction) and utilizes (in the reverse reaction) an entire molecule of poly P without release from the enzyme. Although the poly P from log-phase cells was all long chain and similar to that produced by the poly P kinase, this does not exclude the possibility that other enzymes were present which may have shortened the poly P. It is likely that shortened poly P would be used as a primer (N. A. Robinson and H. G. Wood, Fed. Proc. **45**:1610) by the poly P kinase, thus regenerating the longer chains. From studies of a mutant strain of *Escherichia coli*, it was suggested that the short chains isolated early in growth serve as the primers for synthesis of the long chains which occurred only later in growth (20). In the reverse reaction of the poly P kinase, ATP is synthesized, thereby conserving the energy of the phosphoanhydride bond. The degree to which this reaction occurs physiologically is not known. A role for poly P as an energy reserve has been suggested (13, 14), but it has never been unequivocally demonstrated.

In contrast to growth on lactate, glucose-grown cells did not contain volutin granules and had 100-fold less long-chain poly P than lactate-grown cells, yet the level of the poly P kinase is the same in cells grown on either substrate, as is the level of the poly P glucokinase (32). The amount of long-chain poly P in glucose-grown cells was found to range from 0.4 to 1.4 mM, which is nearly 2 orders of magnitude higher than the K_m of the poly P glucokinase for poly P; for a chain length of 400 residues the K_m is 0.0029 mM, and for 140 residues the K_m is 0.005 mM, in terms of total phosphate (18). In contrast, the ATP level, which generally ranges in bacteria from 1 to 5 mM (12), approaches the K_m value, 1 mM, of the ATP-dependent glucokinase for ATP (unpublished data). The poly P-dependent enzyme, in addition to having an extremely low K_m, has a fourfold-higher specific activity than the ATP-dependent enzyme, suggesting that it predominates (32). We propose that the poly P produced by the poly P kinase is used in glucose-grown cells to phosphorylate the glucose, whereas when lactate is the substrate, the poly P accumulates because there is not an analogous pathway for its utilization. In preliminary studies, we have demonstrated utilization of poly P by whole cells by transferring lactate-grown cells containing a high amount of poly P to fresh glucose medium. During incubation with glucose, the poly P decreased in a manner consistent with the mechanism of the poly P glucokinase reaction (19; C. A. Pepin, N. A. Robinson, and H. G. Wood, Fed. Proc. **45**:1610) such that the amount of extremely long-chain poly P decreased and there was an accumulation on 15% polyacrylamide gels of shorter poly P of about 100 residues in length. In similar studies (28), with *Corynebacterium diphtheriae*, the volutin granules of the cells disappeared during incubation with glucose.

We found that the poly P of lactate-grown cells in the stationary phase was somewhat shorter than that of cells in the log phase. In addition, in contrast to cells in the log phase, the cells in the stationary phase apparently catalyzed shortening of the poly P during the harvesting unless they were first treated with TCA-acetone. Kulaev et al. (15) have reported that a long-chain-dependent polyphosphatase activity is present in older cultures of "*P. shermanii*"; however, the reported activity was low. Harold (5) found with an *Aerobacter aerogenes* mutant deficient in polyphosphatase activity that the amount of poly P decreased at a much slower rate than with wild-type cells and considered that hydrolysis was the main path for utilization of poly P. However, we have been unable to detect polyphosphatase activity in the extracts of "*P. shermanii*" which have the shorter poly P, and we think possibilities other than hydrolysis should be considered. For example, little or no lactate remained when the cells reached the stationary phase, and the poly P may have been utilized to phosphorylate carbohydrate reserves. Propionibacteria grown on lactate produce trehalose (27), and with glucose as the substrate, trehalose was utilized when the glucose was depleted (33). It is likely the trehalose, and possibly the poly P, were utilized when the lactate was exhausted during the stationary phase.

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