Biochemical Studies of Bacterial Sporulation and Germination

XIX. PHOSPHATE METABOLISM DURING SPORULATION*

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SUMMARY

Acid-soluble phosphate compounds represent about 25% of the total phosphate of Bacillus megaterium vegetative cells, but only 7% of the total phosphate in dormant spores. In vegetative cells, 12 compounds make up 90% of the acid-soluble phosphate. These same compounds predominate throughout growth and early sporulation, although their relative amounts change during the process. One hour before refractile spores appear within sporangia, 3-phosphoglyceric acid (PGA) becomes a major component of the sporangial pool and, with the appearance of refractile spores, PGA becomes more difficult to extract. PGA is the largest component of the acid-soluble phosphate pool of B. megaterium spores produced in rich as well as minimal media, and also predominates in Bacillus cereus and Bacillus subtilis spores. Spore PGA appears to be synthesized in a compartment which is inaccessible to inorganic phosphate in the medium, although, at the time of synthesis, \( P_i \), from the medium does enter the sporangium. PGA in dormant spores is very firmly bound, and does not readily exchange with external PGA.

During sporulation, major shifts in metabolism occur. Certain compounds, such as poly-\( \beta \)-hydroxybutyrate, accumulate and are later utilized (1); extensive turnover of macromolecules occurs (2, 3), and some enzyme levels change dramatically (2); new and distinctive spore structures are synthesized, while previously existing structures are degraded (4). Finally, a metabolically inert spore is formed. Are these metabolic changes reflected in the kinds and amounts of small molecules in sporulating cells? Do dormant spores contain ATP, nucleotides, coenzymes, and other intermediates typically found in growing cells?

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The pivotal role of phosphorylated compounds in biosynthetic and energy-yielding reactions led us to compare the pools of low molecular weight phosphate compounds in growing cells with those in spores, and to examine the variations in phosphate pools during sporulation. The earlier studies of Fitz-James (5) and of Yamakawa, Aida, and Uemura (6) indicated that spores contained relatively small amounts of acid-soluble phosphate. We report here significant differences in the kinds of phosphate compounds found in vegetative cells and spores. In an accompanying paper (7), phosphate metabolism during germination is considered. We have also examined pools of free amino acids (8) and sulfur compounds (9) in spores.

EXPERIMENTAL PROCEDURE

Methods

Growth of Cells and Spores—Cells were grown in flasks with vigorous aeration either at 37° in a supplemented nutrient broth (10) or at 30° in a synthetic medium (11) containing sucrose as sole carbon source. The SNB medium, containing 2.0 to 2.5 mM \( P_i \) and about 0.2 mM organic phosphate, was used in all experiments except those in Table IV and Figs. 2 and 3. To improve the uptake of \( ^{32}P \) in these experiments, the \( P_i \) content of the nutrient broth was reduced as follows. Eighty grams of Difco nutrient broth were dissolved in water to give 300 ml of a viscous solution, to which were added slowly (at 45°) first 25 ml of 25% MgCl\(_2\)•7H\(_2\)O and then 1.8 ml of concentrated ammonium hydroxide. A heavy precipitate formed immediately and stirring was continued for 2 hours at 4°. The suspension was centrifuged for 30 min at 10,000 \( \times g \) to remove the bulk of the precipitate, and the supernatant fluid was filtered, under vacuum, through previously washed filter paper. The clear filtrate was diluted with H\(_2\)O to 500 ml. This 20-fold concentrated nutrient broth, when diluted to make SNB, contained 0.21 mM \( P_i \). The amount of NH\(_3\) added in this procedure was not enough to change the final pH of the diluted broth, which was 6.8. Cells of Bacillus megaterium grew in this “low \( P_i \); SNB” medium at the same rate as in SNB, and sporulated normally, but the cell number at the end of exponential growth was lower than that in SNB. \( ^{32}P \) was added to bring the final \( P_i \) concentration to 1.0 mM in the experiments of Table IV and Figs. 2 and 3.

The turbidity of cultures was measured with a Zeiss M4QII

1 The abbreviations used are: SNB, supplemented nutrient broth; PGA, 3-phosphoglyceric acid.
suspended in 5% trichloracetic acid. Two-phase System Y of Sacks and Alderton (13) was used. The methods and sodium dodecyl sulfate, as previously described (12). In gial lysis was not complete, spores were cleaned with lysozyme. Phosphate release nucleic acids. Fitz-James (5) showed that disruption of spores was essential for the proper fractionation of their phosphate pools, as shown under “Results.”

Washing of Cells and Spores—In order to harvest and wash labeled cells rapidly, samples (0.2 to 0.5 ml) of the culture were immediately filtered through 0.45 μm Millipore filters and then washed twice on the filters with 2.0 ml of unlabeled SNB at 15°C. When the filters had been previously soaked in 1 ml of unlabeled SNB, very little background adsorption of 32P occurred. The washed cells on the filter were immediately transferred to a small beaker containing either 2.0 ml of boiling H2O or 2.0 ml of cold 5% trichloracetic acid. Less than 30 sec was required for this washing procedure.

When larger volumes of culture were to be harvested, samples of 5 to 100 ml were chilled 3 min in an ice bath and then centrifuged 3 min at 8000 × g. The supernatant fluid was decanted and discarded. The cell pellet was drained and then quickly suspended in cold 5% trichloracetic acid. A comparison of these two methods of harvesting showed that cells washed on a Millipore filter contained 5 to 100% less P underage than those harvested by centrifugation.

Spores were harvested after maximal sporangial lysis, about 20 hours after the end of growth, and then cleaned in one of three ways. When sporangial lysis was complete and cultures contained no sporangial debris visible in the light microscope, spores were simply washed eight times with cold H2O. After each centrifugation (10 min, 10,000 × g), the light layer at the top of the pellet was rinsed off and discarded. Alternatively, if sporangial lysis was not complete, spores were cleaned with lysozyme and sodium dodecyl sulfate, as previously described (12). In some experiments with Bacillus subtilis, spores were freed of sporangia and debris by partitioning the culture pellet in the two-phase System Y of Sacks and Alderton (13). The method yielded spores uncontaminated by sporangia or detectable debris, and the phosphate content of cleaned spores was the same regardless of the method of cleaning.

Extraction of Cells and Spores—To extract acid-soluble contents, vegetative cells were suspended in 5% trichloracetic acid and subjected to 1 min periods of sonic oscillation, with continuous cooling in an ice bath. The broken cell suspension was held for 30 min at 0°C and then centrifuged for 15 min at 12,000 × g. The supernatant fluid was extracted four times with 2 volumes of ether to remove trichloracetic acid. A second cold trichloracetic acid extraction without sonic treatment released essentially no additional phosphate. The residue was extracted twice with 5% trichloracetic acid at 80°C for 15 min to release nucleic acids. Fitz-James (5) showed that disruption of spores was essential for the proper fractionation of their phosphate compounds. Several methods were therefore tested for their effectiveness in releasing phosphate compounds from dormant spores. Disruption in the Gifford-Wood Mini-mill (14) released a maximum of 25 to 30% of the phosphate from spores of B. subtilis suspended in neutral buffer. This same limit was also reached when disruption was by sonic oscillation with glass beads (8) or by dry rupture (15) or by treatment with lysozyme after exposure to mercaptoethanol and urea (16). These methods all proved unsatisfactory for the analysis of acid-soluble pools, since nuclease activity in the spore extracts released enough nucleotides during the extraction to obscure the true dormant spore pools.

When B. subtilis spores were suspended in 5% trichloracetic acid and then thoroughly disrupted in the Mini-mill (14) at 0-10°C, 5% of their total phosphate was released. However, after extraction of the trichloracetic acid with ether, neutralization with NaOH resulted in the precipitation of 80% of the acid-soluble phosphate, leaving only 1% of the total spore phosphate in the acid-soluble, pH 7-soluble fraction. The material which precipitated at pH 7 was easily dissolved in 5% trichloracetic acid, and, upon removal of the trichloracetic acid with ether, 100% of the 32P was again precipitated. As a control, 14C-ATP was added to intact spores before their disruption in trichloracetic acid. The ATP also showed anomalous solubility properties in that all of the added ATP was recovered in the cold trichloracetic acid extract, but 95% of it was precipitated at pH 7.

Passage of the original cold acid extract through a short column of Dowex 50-Na+ succeeded in removing a factor, perhaps a metal ion, responsible for this precipitation at neutral pH. After such a Dowex treatment, less than 3% of the added ATP and less than 10% of the extracted spore 32P was precipitated at pH 7.

We were concerned that the factors which caused the insolubility at pH 7 of 32P and ATP in the cold acid extracts might also prevent the extraction of normally acid-soluble phosphorus compounds of the spore. To examine this possibility, spores labeled with 32P were extracted by disruption in 5% trichloracetic acid, and the insoluble debris was then suspended and stirred with Dowex 50-Na+ beads. The acid extract contained 5% of the total spore phosphate, and subsequent stirring with Dowex 50 released less than 0.2% of the remaining phosphate. Thus it seems unlikely that there is a significant quantity of small, phosphorus-containing molecules sequestered in the spore as, for example, in metal complexes.

No anomalous solubility properties were observed when spores of B. megaterium were suspended in cold 5% trichloracetic acid and disrupted by sonic oscillation in the presence of glass beads, as previously described (8). This method was therefore used, unless otherwise noted, for the extraction of B. megaterium dormant spore pools.

Treatment of B. subtilis or B. megaterium spores at 100°C for 10 min in either water or neutral buffer caused the complete release of their acid-soluble phosphate pools, as shown under “Results.” It was therefore convenient to use this method to release all PGA from both cells and developing spores in our studies of PGA accumulation during sporulation. After treatment for 30 min in water at 100°C, cell and spore debris was separated from the extract by centrifugation at 12,000 × g for 15 min.

Fractionation and Detection of Acid-soluble Phosphate Compounds—High voltage paper electrophoresis was performed in 20 mM sodium citrate buffer, pH 3.5, on Whatman No. 3MM paper. With the paper immersed in an organic coolant, at 20-25°C, a potential of 125 volts per cm was applied for 25 min. Under these conditions, P1 migrated 30 cm toward the anode.
Mobilities were highly reproducible under these conditions, but substitution of a different grade of paper resulted in changes in mobilities.

Thin layer chromatography in two dimensions on cellulose impregnated with poly(ethyleneimine) was carried out as described (17), with the gradient chromatography method.

Radioactive regions of electropherograms and chromatograms were located by autoradiography as described (9) or by scanning with a Vanguard recording radiation scanner. Unlabeled AMP, CMP, UMP, and GMP were usually mixed with the radioactive samples before electrophoresis or chromatography, and these markers were located as ultraviolet-absorbing spots. Other standard markers were located by spraying the electropherogram with the reagent of Hanes and Isherwood (18).

Isotopic Methods—Carrier-free $^{32}$Pi (0.1 to 5 mCi) was diluted with 0.1 μmole of unlabeled Pi, boiled for 15 min in 1 M HCl, and then neutralized with NaOH immediately before use. Omission of this treatment led, in several instances, to highly anomalous results. Treated $^{32}$Pi was added to culture media to give a specific radioactivity of about 10$^4$ cpm per μmole of Pi. Samples were assayed for radioactivity with a gas flow radiation detector.

Analytical Methods—Pi was determined by the method of Chen, Toribara, and Warner (19). The same method was used for total phosphate, afterashing with Mg(NO$_3$)$_2$ and boiling in 1 M NaOH (19). RNA and DNA were measured colorimetrically after reaction with orcinol (20) or diphenylamine (21). Absorbability to charcoal was tested by mixing a labeled compound with acid-washed Norit A in the presence of carrier Pi, and CMP, at pH 7.0. Compounds adsorbed to Norit were eluted with 50% ethanol containing 3 ml of concentrated NH$_4$OH per 100 ml.

The susceptibility of compounds to hydrolysis by 5'-nucleotidase or bacterial alkaline phosphatase was determined as previously described (22). For the determination of acid lability, a $^{32}$P-labeled compound was treated for 15 min with 1 M HCl at 100°, and liberated $^{32}$Pi was assayed with the reagent of Sugino and Miyoshi (23), which specifically precipitates Pi.

For the specific enzymatic assay of PGA, the compound was incubated with ATP, DPNH, PGA kinase, and glyceraldehyde-3-P dehydrogenase under previously described conditions (24). The stoichiometric conversion of PGA to glyceraldehyde-3-P was obtained by trapping the product with hydrazine, and DPN production was measured by the decrease in absorbance at 340 nm. This assay is specific for the 3-P isomer of PGA; 2-P production was measured by the decrease in absorbance at 340 nm. This assay is specific for the 3-P isomer of PGA; 2-P production was measured by the decrease in absorbance at 340 nm.

Vegetative cells (130 Klett units, Fig. 6) and spores were produced in SNB. Acid-soluble phosphate and nucleic acids were assayed for radioactivity with a gas flow radiation detector.

Fractionation of the acid-soluble extracts of vegetative cells revealed 12 major components (Fig. 1). These included Pi, ATP, CTP, GTP, UTP, DPN, AMP, UMP, ADP, FDP, and α-glycerophosphate. The ratio of ATP to AMP and ADP was lower than expected at myokinase equilibrium, suggesting that some ATP breakdown occurred during extraction. Pi was the predominant component of the acid-soluble fraction, and the other compounds occurred in amounts ranging from 0.2 to 20

**Table I**

**Phosphate distribution in vegetative cells and spores**

| Distribution in          | Vegetative cell | B. megaterium | B. subtilis* |
|-------------------------|-----------------|---------------|-------------|
| RNA                     | 50              | 35            | 35          |
| DNA                     | 9               | 8             | 10          |
| Acid-soluble            | 29              | 7             | 3           |
| Lipid                   | 2               | 2             | 2           |
| Residual                | 10              | 18            | 48          |
| Dry weight (%) as phosphorus | 1.6           | 1.6           | 1.6         |
| Phosphate per cell or spore (10$^{-10}$ mole) | 2.2 | 0.50 | 0.17 |

*The sum of all fractions listed for B. subtilis is only 93%; the acid-insoluble, non-nucleic acid fraction described in the text accounts for the remainder.
nmoles per ml of culture, or about 0.1 to 10 μmoles per g of dried cells. Together, these 12 compounds represented more than 90% of the acid-soluble phosphate of vegetative cells.

Distribution of Phosphate Compounds in Spores—Spores of *B. megaterium* and *B. subtilis* contained 1.0 to 1.6% of their dry weight as phosphorus, but the distribution was quite different from that found in vegetative cells. Nucleic acids made up 45% of the total phosphate, but the acid-soluble fraction was only 3 to 7% in spores (Table I). A fraction resistant to extraction with hot trichloracetic acid (the "residual fraction") made up 40 to 50% of the total phosphate in spores of both species (Table I).

**Acid-soluble Phosphate Compounds**

The acid-soluble phosphate extracted from *B. megaterium* spores was resolved into at least nine components by paper electrophoresis (Fig. 2). Two of these components made up more than 90% of the extracted phosphate. One, containing 20% of the acid-soluble phosphate, was identified as Pi. The second major species, slightly more acidic than Pi at pH 3.5, represented about 75% of the extracted phosphate.

**PGA as Predominant Acid-soluble Phosphate Compound in Spores**—The most abundant acid-soluble phosphate compound in spores was purified by chromatography on Dowex 1, from which a peak of constant specific radioactivity was obtained. The purified compound had the same electrophoretic mobility at pH 3.5 or 5.0 as phosphoglyceric acid. It reacted with 4,5-dihydroxy-2,7-napthalene disulfonic acid (chromotropic acid) under defined conditions (25), yielding a product with the same absorption spectrum and extinction coefficient as glyceraldehyde and its phosphomonoesters. The intact compound was insensitive to periodate but, after the removal of phosphate with bac-

**TABLE II**

**Identification of acid-soluble phosphate compounds in vegetative cells of *B. megaterium***

Acid-soluble components of vegetative cells, extracted and separated as in Fig. 1, were eluted and characterized by adsorbability to Norit, susceptibility to hydrolysis by 5'-nucleotidase, lability to acid (1 M HCl, 10 min, 100°C), and paper electrophoresis at pH 3.5. Each compound listed coelectrophoresed with the appropriate unlabeled marker.

| Compound | Amount (μmoles/g cells) | Norit adsorbability | Other |
|----------|-------------------------|---------------------|-------|
| Pi   | 24.6                     | -                   | Chemical determination |
| DPN  | 1.2                     | +                   | Enzymatic assay |
| AMP  | 1.6                     | +                   | Sensitive to 5'-nucleotidase |
| ADP  | 0.4                     | +                   | 50% acid-labile |
| ATP  | 2.8                     | +                   | 60% acid-labile |
| CTP  | 0.6                     | +                   | 65% acid-labile |
| GTP  | 0.5                     | +                   | 63% acid-labile |
| UTP  | 0.2                     | +                   | 60% acid-labile |
| UMP  | 0.4                     | + Sensitive to 5'-nucleotidase |
| CMP  | 0.1                     | + Sensitive to 5'-nucleotidase |
| FDP  | 9.0                     | - 50% acid-labile |
| α-Glycerol-P | 3.6 | - | Acid-stable |

**Fig. 2. Electrophoretic separation of acid-soluble phosphate compounds from spores. *B. megaterium* spores produced in "low Pi, SNB" containing □P_1 (1 mM) were extracted into cold trichloroacetic acid by sonic disintegration (see "Methods"). Neutralized extracts were subjected to electrophoresis at pH 3.5. The patterns above were obtained by scanning the electropherogram for radioactivity at two levels of instrument sensitivity. Radioactivity is represented on the ordinate scale, and the positions of unlabeled markers are indicated by arrows.**
terial alkaline phosphatase, periodate liberated 1 mole of formaldehyde per mole of phosphate originally present. The infrared spectrum of the compound from spores was identical with that of authentic 3-P-glyceric acid.

Glyceraldehyde-3-P dehydrogenase and PGA kinase together catalyzed the oxidation of DPNH in the presence of the spore compound, ATP, and Mg++. When hydrazine was used to trap the glyceraldehyde-P and drive the reaction to completion, 1.0 eq of DPN was produced per phosphate. Under exactly these assay conditions only 3-phospho-D-glyceric acid, and not the 2 isomer, served as substrate. These analyses, summarized in Table III, establish the identity of the spore compound as 3-P-D-glyceric acid. Spores of *B. subtilis*, *B. cereus*, and *Bacillus thuringiensis*, produced in SNB medium, also contained PGA as the major component of their acid-soluble pools (26), as did *B. megaterium* spores produced in SNB containing P_i at a growth-limiting concentration (0.2 mM) or in a synthetic (11) medium.

**Less Abundant Acid-soluble Phosphate Compounds in Spores**—Minor components of *B. megaterium* spore extracts (Fig. 3) have been tentatively identified, on the basis of criteria summarized in Table IV, as AMP, ADP, CMP, GMP, UMP, glycerol-P, and DPN. Each of these components represents less than 5% of the acid-soluble phosphate of spores.

The levels of ATP and other nucleoside triphosphates in spores of *B. megaterium* and *B. subtilis* were too low to detect by the methods used here. When ^3^C-ATP was added to dormant spores before the extraction of their acid-soluble pools, more than 90% of the ^3^C was recovered in the extracts as ATP. Yet, when extracts of ^32^P-labeled spores were fractionated by paper electrophoresis or anion exchange column chromatography, essentially no ^32^P was associated with the ^3^C-ATP. Furthermore, when spore extracts were fractionated by thin layer chromatography as in Fig. 3, autoradiographic exposures 50 times longer than that required to show AMP revealed no radioactivity associated with unlabeled ATP marker. Thus, spores contain less than 10 nmoles of ATP per g, dry weight, and the same is true for other nucleoside triphosphates as well.

**Stability of Acid-soluble Phosphate Compounds in Dormant Spore**—Repeated washing with water at 4° caused no detectable release of PGA. Spores stored for several weeks as a frozen

### Table III

**Identification of 3-P-D-glyceric acid in spores**

| Analysis                                      | Result                                                                 |
|-----------------------------------------------|----------------------------------------------------------------------|
| Paper electrophoresis, pH 3.5, 5.0            | Coelectrophoresis with P-glyceric acid                               |
| Reaction with chromotropic acid (25)         | Same visible absorption spectrum and ε as 3-P-glycerate product      |
| Periodate after phosphatase                   | Periodate liberates 1.0 eq of formaldehyde per phosphate            |
| Infrared absorption spectrum                  | Spectrum identical with that of 3-P-glyceric acid                    |
| Enzymatic conversion to glyceraldehyde-3-P   | 1.0 pmole of DPN produced per pmole of phosphate; reaction specific for 3 isomer |

### Table IV

**Identification of acid-soluble phosphate compounds in spores of *B. megaterium***

Spores were produced in "low P_i SNB" supplemented with ^32^P (final P_i concentration was 1 mM). Acid-soluble components of spores, extracted and separated as in Fig. 3, were eluted and characterized as in Table II.

| Compound                       | Amount | Nont labile | Other |
|-------------------------------|--------|-------------|-------|
| 3-P-Glycerate                 | 15.6   | —           | Table III |
| P_i                           | 4.2    | —           | Chemical determination |
| CMP                           | 0.2    | +           | Sensitive to 5'-nucleotidase |
| AMP                           | 1.2    | +           | Sensitive to 5'-nucleotidase |
| GMP                           | 0.2    | +           | Sensitive to 5'-nucleotidase |
| UMP                           | 0.1    | +           | Sensitive to 5'-nucleotidase |
| ADP                           | 0.3    | +           | 50% acid-labile |
| DPN                           | 0.0-0.2 | +           |       |
| Glycerol-P                    | 0.2    | —           | Acid-stable |
| ATP, CTP, UTP, GTP            | <0.01  | —           | Position on thin layer chromatography |
| 2,3-Diphosphoglycerate        | <0.01  | —           | Position on thin layer chromatography |

*Ex except in the case of nucleoside triphosphates and 2,3-diphosphoglyceric acid, the identity of each compound was verified by its coelectrophoresis with unlabeled marker at pH 3.5.

* Values for DPN varied, presumably because of its instability in extracts. A heat-activated DPNase such as that found in other bacilli (27) may be present in hot water extracts of spores.

![Fig. 3. Thin layer chromatographic separation of acid-soluble phosphate compounds from spores. Extracts of labeled spores were prepared as in Fig. 2 and fractionated as in Fig. 1.](image-url)
suspension contained essentially unaltered patterns of phosphate compounds (26), with one exception; there was a slow release of P\(_1\) during extended storage of B. subtilis and B. thuringiensis spores. No PGA was released from dormant spores held in 5% trichloracetic acid at 0° for 2 hours, although, as shown above, the compound was readily extracted from spores disrupted by sonic oscillation. PGA was released from spores at 93° (in water) after 20 to 30 min (26).

Exchange of PGA in B. megaterium spores (10 mg containing about 0.15 μmole of PGA) with external \(\text{P}^3\) PGA (0.2 μmole in 0.5 ml of 50 mM Tris-HCl, pH 7.8, for 9 hours at 4°) was less than 3% (4 nmoles). Similarly, spores took up less than 6% as much external \(\text{P}^3\) as the \(\text{P}^3\) in the spores. No germination occurred in these experiments. The failure of exogenous \(\text{P}^3\) or PGA to exchange with endogenous pools of these compounds points to their relative inaccessibility in the dormant spore. Free glutamate in spores was also nonexchangeable (8).

Acid-insoluble Phosphate Compounds in Spores—DNA and RNA made up about 45% of the total spore phosphate in B. subtilis and B. megaterium (Table I). The amount of nucleic acid per cell or spore and the ratios of RNA to DNA were in good agreement with other published values (5, 12, 28). Spores of both species contained about half of their phosphate in a form not extractable under conditions normally used to extract nucleic acids. Spores held at 100° in neutral buffer released a maximum of 7 to 12% of their total phosphate (Fig. 4), including PGA. Treatment of intact spores with 5% trichloracetic acid at 100° quickly released 50 to 60% of their total phosphate, but further treatment caused little or no additional phosphate release (Fig. 4). Similarly, 0.1 M NaOH at 100° extracted 55 to 60% of the spore phosphate within 5 min, but longer treatment gave no more release (Fig. 4). This “resistant residue” fraction was described by Fitz-James (5) and appears to be associated with spore coats. Although the exact composition of residue phosphate is not known, there is a report (29) that, in B. megaterium spores, phosphomuramic acid is present.

When B. subtilis spores were mechanically disrupted by Mini-mill treatment, almost all of the phosphate in the water extract was insoluble in cold 5% trichloracetic acid. The acid-insoluble fraction of this extract consisted chiefly of nucleic acid, but it also contained a non-nucleic acid component, representing about 8% of the total spore phosphate. This fraction did not dissolve in neutral buffer after precipitation with trichloracetic acid, whereas the nucleic acid dissolved readily. The fraction did dissolve in 1 mM NaOH, giving a viscous yellow solution. The absorption spectrum of this solution showed no maximum in the region 240 to 310 μm; the extinction at 260 μm and analyses for phosphate uptake was determined by measuring radioactivity on the washed filters. Acid-soluble phosphate in the B. megaterium culture was determined by extracting washed cells immediately with cold trichloracetic acid (see “Methods”).
ribose and deoxyribose indicated a nucleic acid content of less than 20% of the phosphate. This component has not been identified although some of its properties suggest that it may be a cell wall polymer, derived, perhaps, from the spore coat.

Variations in Phosphate Content of Cells during Growth and Sporulation—The uptake of \( P_i \) from the medium paralleled growth in both *B. megaterium* and *B. subtilis*, reaching a maximum at the end of exponential growth (Fig. 5). In the following 2 hours, there was a rapid loss of about 10% of the total phosphate, after which the phosphate content remained nearly constant for 2 hours. At about the time that refractile spores appeared, but before any sporangial lysis was apparent, another rapid decline in phosphate content began. Twenty-five hours after inoculation, both cultures contained more than 90% free spores, with a phosphate content of about 40% of the amount in cells at the end of growth. Washing the spores reduced this value to about 25% of the amount in vegetative cells.

Acid-soluble phosphate represented about 20% of total phosphate throughout the growth and sporulation of *B. megaterium*. The amount of acid-soluble phosphate reached a maximum after the end of exponential growth, and then remained nearly constant for 4 hours before decreasing along with the total phosphate. Electrophoretic fractionation of these acid-soluble fractions revealed no major qualitative changes in the patterns of these compounds during growth and sporulation. There were, however, significant changes in the relative amounts of these components during sporulation, including an increase in the level of PGA.

**PGA Synthesis during Sporulation**

*Kinetics of PGA Accumulation*—PGA accumulation began about 1 hour before the appearance of refractile spores (Fig. 6). A significant fraction of the total PGA in sporulating cells was not extracted by suspending cells in 5% trichloracetic acid without sonic disruption; its release required treatment at 100° in water. This “inaccessible” fraction of PGA accumulated at the same time as refractile spores appeared (Fig. 6). At about the time of sporangial lysis, the level of PGA decreased by about 50%, leaving only the “inaccessible” fraction, which was localized in the spores. PGA in the culture medium was not determined. The low and constant level of PGA present in hot water extracts of growing cells and during early sporulation may be an artifact of the hot water extraction. Essentially no PGA was found in

![Fig. 6. Variation in PGA during sporulation. *B. megaterium* cells growing in \(^{32}P\)-labeled SNB were harvested and washed by Millipore filtration and then extracted either with cold 5% trichloracetic acid (TCA) (30 min, 4°, no sonic oscillation) or with water (30 min, 100°). Radioactive PGA was assayed after electrophoretic separation. This assay does not distinguish between 2-P-glycerate and 3-P-glycerate.](#)

![Fig. 7. Time of labeling of spore phosphate compounds. Six identical cultures of *B. megaterium* were grown in \(^{32}P\)-labeled SNB (1.2 × 10^6 cpm per mole of \( P_i \)). At the times indicated by arrows, one-tenth of the culture was removed. Cells were harvested by centrifugation and extracted with cold trichloracetic acid. PGA in each extract was determined as in Fig. 6; the time of PGA accumulation is shown here. Immediately after the removal of each culture sample, potassium phosphate buffer (pH 7.4, unlabeled) was added to the culture to give a \( P_i \) concentration of 50 mM, about 25 times greater than the original \( P_i \) concentration in the medium. After this “chase,” each culture was allowed to sporulate and, at 27.5 hours, all cultures contained 90% or greater free spores. Direct counts showed that the number of spores per ml was the same (within 10%) in all cultures. Hot water extracts of spores from four “chased” cultures and one unchased control were prepared and analyzed for total PGA (enzymatic assay) and for radioactive PGA (electrophoretic assay). The final specific radioactivity was calculated from these determinations, and is plotted here as a function of the time that unlabeled \( P_i \) was added. The specific radioactivity of free \( P_i \) in mature spores was similarly determined, from chemical and isotopic measurements of \( P_i \). The time of spore coat phosphate synthesis was determined in the sixth parallel culture. Samples of the culture were centrifuged, and cells were extracted for 20 min at 100° with 5% trichloracetic acid. This treatment solubilized almost all vegetative cell phosphate but left the spore coat phosphate insoluble (Table I). The specific radioactivity of spore coat phosphate was determined in mature spores from cultures chased at different times, and varied as shown here.](#)
cold trichloroacetic acid extracts during this period, although cold trichloroacetic acid extraction without sonic disruption gave virtually complete release of ATP and DPN.

Forespore as Probable Site of PGA Synthesis and Impermeability of Forespore—We attempted to chase the $^{32}$P-label from previously labeled P-glycerate by adding unlabeled $P_i$ at intervals during PGA accumulation (Fig. 7). A 25-fold excess of unlabeled $P_i$, added to the medium 1 hour before the first refractile spores appeared, when PGA synthesis had not yet begun, caused a dilution of only 37% in the specific radioactivity of PGA isolated from mature spores. Similar chases at later times, when 7, 25, and 75% of the cells contained refractile spores, resulted in less than a 5% decrease of the PGA specific radioactivity in mature spores, compared with that in a culture with no chase. The $P_i$ isolated from spores chased at the same times showed a 5-fold decrease in specific radioactivity with the earliest chase, but progressively less dilution in specific radioactivity occurred when the chase came later (Fig. 7). These results suggest that the forespore compartment at this stage is relatively inaccessible to $P_i$ in the medium.

Nonetheless, $P_i$ does enter the sporangium, and is incorporated into the outer layers of the spore, when added to the medium even at these later stages of sporulation. Insolubility in hot acid was used to define the coat phosphate fraction and to determine the time of its synthesis. At least part of the coat phosphate fraction was synthesized quite late, about 2 hours after PGA synthesis was completed (Fig. 7). In contrast to the results with PGA, the phosphate in this coat fraction was diluted by $P_i$ added to the medium late in sporulation (Fig. 7).

**DISCUSSION**

**Spore Pool of Acid-soluble Phosphate Is Unique**—The absence of metabolic activity in dormant spores is reflected in their acid-soluble phosphate compounds, which differ strikingly both in amount and in kind from those of vegetative cells. Spores contain only 3 to 7% of the phosphate as acid-soluble compounds, a fraction appreciably lower than the 20 to 30% present in vegetative cells and sporangia. Even more significant are the differences in the compositions of the acid-soluble phosphate pools. Spores contain very low levels of ATP, whereas ATP is a major component of vegetative cells. On the other hand, 3-P-glyceric acid constitutes as much as 75% of the acid-soluble phosphate in the spore, but no more than 5% of the acid-soluble phosphate of the vegetative cell. PGA is present at these high levels in *B. megaterium* spores produced in a rich or minimal medium, and is also the predominant component in the acid-soluble phosphate pools in spores of three other species, *B. cereus*, *D. subtilis*, and *B. thuringiensis* (26).

Spores contain, in addition to PGA and $P_i$, much smaller amounts of other acid-soluble phosphate compounds, including DPN, the nucleoside 5'-monophosphates, ADP, and glycerophosphate. Some of these less abundant spore components are similar to or identical with the major acid-soluble phosphate compounds of vegetative cells. Moreover, the levels of these components (e.g., DPN) are comparable in vegetative cells and spores, when expressed in terms of cell or spore dry weight; a more detailed analysis of adenine, pyridine, and flavin nucleotides in spores and vegetative cells will be presented elsewhere.2

**PGA May Be Made in Forespore**—Our observations of a slight increase in acid-soluble phosphate after the end of growth and a sharp decrease during sporangial lysis agree with those of Yamakawa et al. (6). Their “sugar-P” fraction, which should have included PGA, increased about 2-fold during the period of spore formation, and then decreased with sporangial lysis. We have shown that the amount of PGA eventually found in spores is about half of the total amount accumulated in sporangia, the remainder being lost during sporangial lysis, perhaps by release to the medium. PGA became inaccessible to direct extraction with trichloroacetic acid at the time that refractile spores appeared. The large dilution of $^{32}$P in the spore coat phosphate, by $P_i$ added to the medium indicates access of exogenous $P_i$ to the mother cell compartment. In contrast, the relatively slight dilution of $^{32}$P in PGA of the spore, when a large excess of exogenous $P_i$ was added at the onset of PGA synthesis, suggests that PGA is synthesized from a precursor pool distinct from that of the mother cell, perhaps within the forespore. Alternatively, the size of the pool of phosphorylated precursors for PGA synthesis may be large compared with the pool of spore coat precursors. The fact that the internal $P_i$ pool was diluted less than coat phosphate makes this alternative less attractive to us.

It is very difficult physically to separate the forespore from its mother cell and thus to compare the metabolism in the two compartments. The preferential extraction of small metabolites from one of the two compartments and the different accessibilities of the compartments to the external medium may possibly offer means of exploring metabolism in the forespore compartment even in intact sporangia.

**Role of Acid-soluble Pool in Spores**—The role of small phosphate compounds, and particularly of PGA in spores, is not clear. These compounds could be metabolic byproducts of sporulation, without significance for the maintenance of dormancy or the mechanics of germination. However, the similarity in distribution of phosphate compounds in spores of different species, produced in different media, suggests that this particular collection of small molecules is in some way essential to the spore. Clearly, coenzymes such as pyridine and adenine nucleotides are needed during germination, when metabolic activity sets in rapidly. Of particular interest to us is the possibility that PGA serves as a ready source of energy in the earliest stages of germination. It is far more stable chemically than ATP, and can yield ATP via phosphoenolpyruvate without the expenditure of energy. The amount of PGA in spores, 10 to 15 amoles per g, dry weight, is actually 3 to 5 times greater than the amount of ATP in vegetative cells. The possibility of PGA serving as an “ATP reservoir” in the spore is explored further in the following paper (7).

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