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Methanophosphagen: Unique cyclic pyrophosphate isolated from *Methanobacterium thermoautotrophicum*

(methanogens/^{31}P NMR/2,3-cyclopyrophosphoglycerate/phosphagen)

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ABSTRACT **A unique cyclic pyrophosphate compound has been detected at 10–12 mM intracellular concentration in *Methanobacterium thermoautotrophicum* by *in vivo* ^{31}P NMR. This compound has been extracted from cells and purified by anion-exchange chromatography. Studies with ^{1}H, ^{13}C, and ^{31}P NMR and fast-atom-bombardment mass spectrometry have identified it as 2,3-cyclopyrophosphoglycerate, an intramolecularly cyclized pyrophosphate of 2,3-diphosphoglycerate. Chemical degradation to 2,3-diphosphoglycerate and synthesis by dicyclohexylcarbodiimide coupling of 2,3-diphosphoglycerate are consistent with this identification. It is suggested that this compound serves as a primary phosphagen in methanogens.**

Methanogens are fastidious anaerobes whose metabolism centers around the reduction of carbon dioxide to methane (1–3). Methanogenesis appears to be an ancient archaebacterial phenotype with unique biochemistry. It differs dramatically from well-known metabolic pathways and may have arisen separately from them. Little is known about the bioenergetics and detailed metabolic pathways in this unusual microorganism.

In vivo ^{31}P NMR is a useful noninvasive technique for monitoring steady-state levels of phosphorus-containing metabolites (4–6). *Escherichia coli* (7, 8), yeast (9, 10), and mammalian cells (11–13) have been studied in detail with this technique. ^{31}P NMR also has been applied to metabolic studies of anaerobic thermophiles such as *Clostridium thermocellum* (unpublished data). Information about intracellular pH, Mg^{2+} concentration, glycolysis intermediates, ATP/ADP charge, and unusual phosphorus-containing compounds can be obtained readily. We used this approach to study phosphorus metabolites of *Methanobacterium thermoautotrophicum*. It allowed us to identify and isolate "methanophosphagen," a unique cyclized pyrophosphate of 2,3-diphosphoglycerate from that unusual organism. We characterized it by spectroscopic techniques, specifically degraded it to 2,3-diphosphoglycerate, and synthesized it by dicyclohexylcarbodiimide coupling of dilute solutions of 2,3-diphosphoglycerate. The compound is suggested to be a new phosphagen in methanogens. Evidence to support this theory is derived from ^{31}P NMR spectra of cell extracts.

MATERIALS AND METHODS

Chemicals. The following chemicals were used without further purification: $Ba(OH)_2$ (Fisher); dicyclohexylcarbodiimide (Aldrich); Pipes, 2,3-diphosphoglycerate, 2-phosphoglycerate, 3-phosphoglycerate, $NADP^+$, NADPH, NAD^+, NADH, UDPG, coenzyme A, FAD, and FMN (all from Sigma); $NaBH_4$ (Alfa–Ventron, Danvers, MA); $Na_2S_2O_4$ (Sigma); and QAE-Sephadex (Sigma).

Cell Growth. *M. thermoautotrophicum* cells were grown as described by Zeikus and Wolfe (14) in a modified medium containing 10 mM inorganic phosphate (instead of 4 mM phosphate) and 50 mM Pipes, pH 7.2 (at room temperature). Cells were grown anaerobically at 62°C in a Shaker bath with feeding of H_2/CO_2, 4:1 (vol/vol), at 18–20 psi (1 psi = 6.89 kPa). Cells were harvested anaerobically and washed with medium containing no paramagnetic ions. The cell paste was then stored at −20°C or resuspended in medium free of transition metal ions for *in vivo* ^{31}P NMR spectroscopy. This latter cell suspension was energized by pressurizing with H_2/CO_2.

Cell Extracts. Two procedures were used to produce cell extracts enriched in small-molecule phosphorus-containing compounds: ethanol extraction and perchloric acid extraction (15).

For ethanol extraction, 4 g of frozen cell paste was suspended in 100 ml of cold ethanol/H_2O, 7:3 (vol/vol), and stirred vigorously under a nitrogen atmosphere for 20 min. The lysed cell suspension was centrifuged at 10,000 rpm in an SS-34 rotor (Sorvall) for 115 min, and the supernatant was removed. Ethanol (50 ml of the ethanol/H_2O solution) was again added to the pellet for a second extraction. After centrifugation the supernatant from this extract was combined with the original supernatant. Solvent was removed by rotary evaporation, and the residue was dried by lyophilization.

For $HClO_4$ extraction, 5 g of frozen cell paste (or the equivalent of an NMR sample) was suspended in 100 ml of deionized water and treated with 3 ml of cold 70% $HClO_4$. The cell suspension was stirred for 20 min; it was kept cold by periodically immersing the sample in an acetone/dry ice bath. Cell debris was removed by centrifuging at 10,000 rpm in an SS-34 rotor (Sorvall) for 10 min. The supernatant was separated and neutralized with 5 M KOH; the solution was then centrifuged to remove insoluble $KClO_4$.

Phosphorus compounds were identified by spiking extracts (pH 6–8) with authentic material.

QAE-Sephadex Chromatography. Both acid and ethanol extracts of methanogens were chromatographed on QAE-Sephadex resin. Samples (85–115 ml) were applied to a 1.5 × 45 cm column equilibrated with 50 mM ammonium acetate (pH 7.5). The novel pyrophosphate compound was eluted by a linear gradient of 0.3–1.5 M ammonium acetate (pH 7.5). Fractions (5 ml) were collected and monitored for absorbance at 430 nm (to detect F_{420} and F_{430} cofactors present in methanogens) and at 260 nm to detect nucleotides and related compounds. Fractions were also assayed for phosphorus content by colorimetric quantitation of inorganic phosphate after $HClO_4$ digestion (16). Fractions containing phosphorus were pooled, and these discrete samples were analyzed by ^{31}P NMR spectroscopy. For further characterization studies, the pooled samples were lyophilized to remove the ammonium acetate buffer.

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Abbreviation: FAB, fast atom bombardment.

NMR Spectroscopy. ^1H (270 MHz), ^1H-noise-decoupled ^{13}C (67.9 MHz), and ^{31}P (109.3 MHz) NMR spectra were obtained with a Bruker 270 spectrometer equipped with a Nicolet 1080 computer. Both *in vivo* and *in vitro* spectra were obtained without sample spinning. Typical *in vivo* ^{31}P spectral parameters include 8,000-Hz sweep width, 60° pulse, and 0.512-sec recycle time. ^{31}P homonuclear decoupling at 109.302259 MHz was used to show that the ^{31}P four-line pattern of the new pyrophosphate was caused by ^{31}P–^{31}P coupling.

FAB Mass Spectrometry. Low-resolution mass spectra and a high-resolution exact mass measurement were made by using a Varian MAT 731 mass spectrometer filled with a modified Ion Tech neutral atom gun (17). The samples were dissolved in glycerol, and approximately 1 μl was placed on the fast-atom-bombardment (FAB) probe tip (18). The FAB source operated with xenon as the neutral atom beam.

RESULTS

***In Vivo* and *in Vitro* ^{31}P NMR Spectroscopy.** *In vivo* ^{31}P NMR spectra at 60°C of the methanogens (at cell densities such that the internal cell volume was one-fifth the sample volume) in the resting state (under H_2) and in the energized state (under H_2/CO_2, 4:1, vol/vol) showed resonances in two main phosphorus regions: inorganic phosphate at −2.8 ppm from the buffer medium and two peaks centered at 9.3 ppm separated by 1.1 ppm (in the pyrophosphate region) (Fig. 1). Comparison of the intensity of the pyrophosphate peaks with that of inorganic phosphate suggests that the concentration of each of these phosphorus groups is 10–12 mM. No other phosphorus metabolites were easily observed by *in vivo* ^{31}P NMR spectroscopy. When the cells were suspended in a phosphate-free buffer, the pyrophosphate resonances dominated the spectrum (data not shown), and a small peak in the inorganic phosphate region indicated that the internal pH was about 7.0.

In ^{31}P NMR spectra of ethanol and perchloric extracts, the pyrophosphate region was resolved into an AB quartet pattern with traces of other phosphate metabolites (Fig. 2). Homonuclear ^{31}P decoupling experiments performed by selectively irradiating the upfield phosphorus doublet showed a decrease in the coupling constant of the downfield pair, confirming that the AB quartet is produced by two asymmetric phosphorus nuclei arranged in a pyrophosphate linkage. The chemical shifts of this pyrophosphate were insensitive to pH over the range 4–9, suggesting that both phosphates are esterified. A comparison of the ^{31}P chemical shifts of the unknown pyrophosphate with the known pyrophosphate esters $NAD(P)^+$, NAD(P)H, FAD, UDPG, and coenzyme A (Fig. 3) indicates that the methanogen pyrophosphate is chemically distinct from these compounds.

Treatment of the extracts with reducing agents such as sodium borohydride or sodium dithionite caused loss of 420 nm absorbance (due to the reduction of a deazaflavin cofactor) but

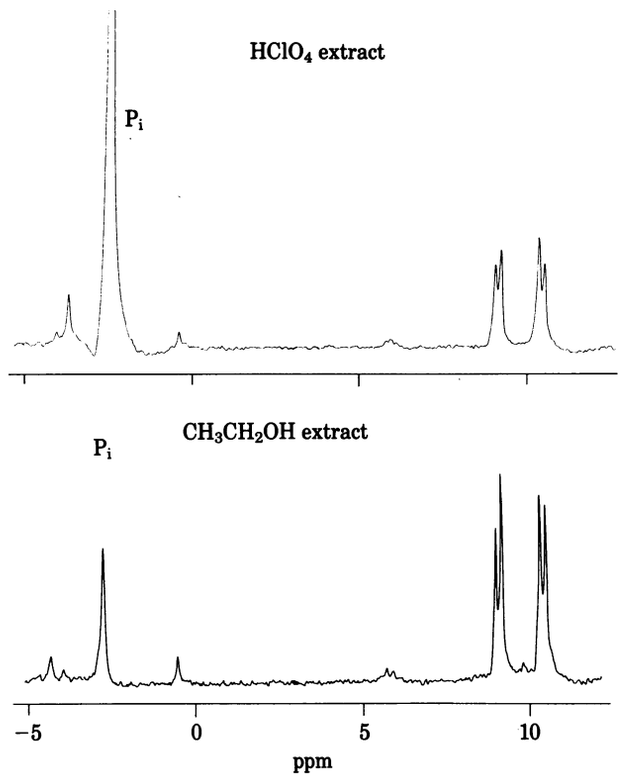


FIG. 2. ^{31}P NMR spectra (109.3 MHz) of a perchloric acid extract (*Upper*) and an ethanol extract (*Lower*) of *M. thermoautotrophicum* in ^2H_2O at pH 7.5. NMR parameters are the same as in Fig. 1 with the exception of a 1-sec recycle time. The four peaks centered at 9.7 ppm are assigned to a novel cyclic-pyrophosphate compound.

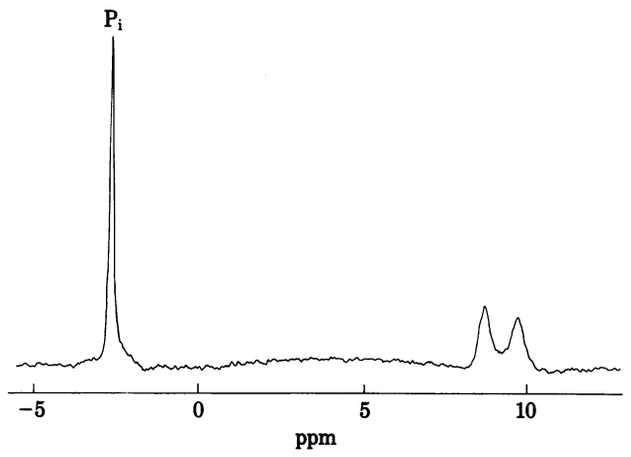


FIG. 1. ^{31}P NMR spectrum (109.3 MHz) of *M. thermoautotrophicum* (0.4-ml cell paste in 2-ml sample) suspended in 10 mM potassium phosphate/100 mM Pipes/37 mM NH_4Cl/2 mM $MgCl_2$/2.6 mM Na_2CO_3/2 mM Na_2S, pH 7.2. The NMR buffer has 50% ^2H_2O and none of the metal elixir used in the growth medium. NMR parameters include 1,000 transients, a 60° pulse width, and 0.512-sec recycle time.

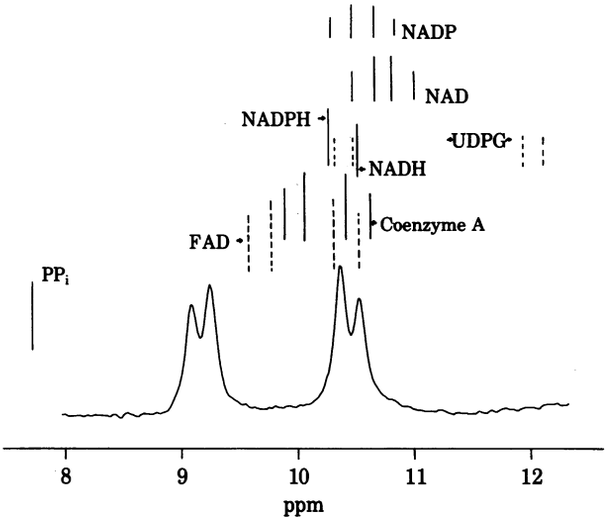


FIG. 3. Comparison of ^{31}P chemical shifts of the unknown pyrophosphate AB quartet with known pyrophosphate resonances. Chemical shifts are referenced with respect to external 70% H_3PO_4.

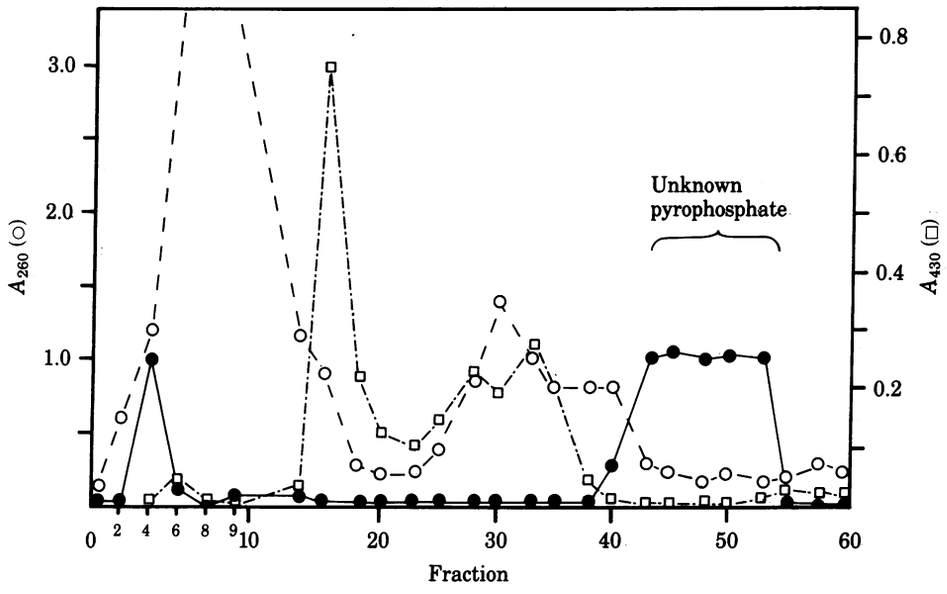


FIG. 4. QAE-Sephadex column elution profile of an ethanol extract of *M. thermoautotrophicum*, showing absorbance at 260 (○) and 430 (□) nm. ●, Absorbance at 660 nm in a colorimetric assay monitors biological phosphate content; the major phosphorus peak at fractions 38–54 (≈0.7 M ammonium acetate) was pooled and used in subsequent analyses.

no alteration in the ^{31}P chemical shifts of the pyrophosphate compound. Attempts to hydrolyze the pyrophosphate linkage with phosphodiesterase I (Sigma) containing known pyrophosphatase and nucleotidase activity failed.

Purification and Spectral Characterization of the Novel Pyrophosphate. Fig. 4 shows the profile of an ethanol extract fractionated on the QAE-Sephadex column run with a 0.3–1.5 M ammonium acetate gradient. Several phosphorus-containing peaks are visible, with the major one eluting with 0.7 M salt. These fractions were pooled, lyophilized, and resuspended in ^2H_2O; they showed no detectable absorbance at 430 and 260 nm but did show the ^{31}P NMR AB quartet pattern indicative of the novel pyrophosphate. A ^1H-noise-decoupled ^{13}C NMR spectrum of this purified fraction showed only three different types of carbon in the compound, all coupled to ^{31}P. A ^1H-coupled ^{13}C NMR spectrum identified the peak centered at 77.86 ppm with a carbon-phosphorus coupling constant (J_{CP}) of 9 Hz as that of a methine carbon, the peak centered at 69.39 ppm (J_{CP} = 7 Hz) as a methylene carbon, and the doublet centered at 173.74 ppm (J_{CP} = 11 Hz) as a carboxylate carbon. Comparison of the carbon chemical shifts of the novel pyrophosphate with those of 2,3-diphosphoglycerate (CH_2O, 66.43 ppm; CHO, 75.21 ppm; COO^-, 178.76 ppm) suggests similar environments for each carbon.

^1H NMR spectra of the isolated material showed two types of protons [4.20 and 4.76 ppm referenced to 3-(trimethylsilyl)-tetradeutero sodium propionate (TSP)]: a complex multiplet upfield of $^1H^2HO$, presumably due to nonequivalent methylene protons coupled to ^{31}P, each other, and the methine proton; and a multiplet downfield of and partially overlapping with $^1H^2HO$ (the CHOP group).

Based on this spectroscopic evidence, the structures shown in Fig. 5 are possible candidates for the novel pyrophosphate. Mass spectrometry can be used to choose between these structures. In the FAB mass spectrometric experiments, one generally observes molecular protonated or cationized ions—e.g., $(M + H)^+$, $(M + Na)^+$, $(M - H + 2Na^+)$—whose abundance depends on the nature of the compound and the amount and types of salts present. The low-resolution spectrum of the novel pyrophosphate yielded at least four sodium adducts with the dominant species appearing at a normal mass-to-charge ratio

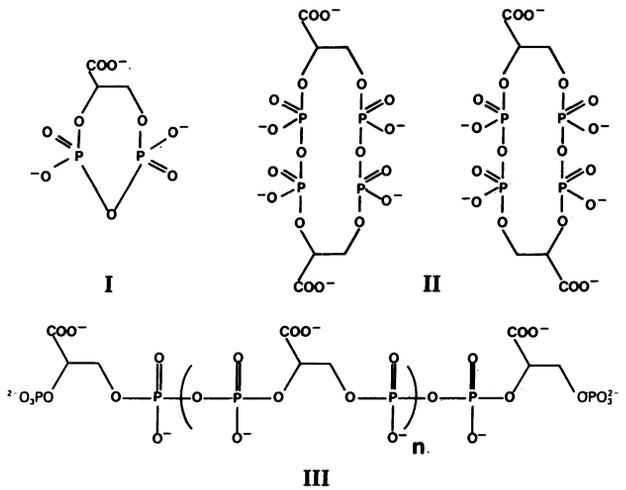


FIG. 5. Possible structures of the novel pyrophosphate compound: cyclized 2,3-diphosphoglycerate (structure I), dimeric pyrophosphates of 2,3-diphosphoglycerate (structure II), and a linear pyrophosphate polymer of 2,3-diphosphoglycerate (structure III) with *n* large because the end phosphate groups are not seen in the ^{31}P NMR spectrum.

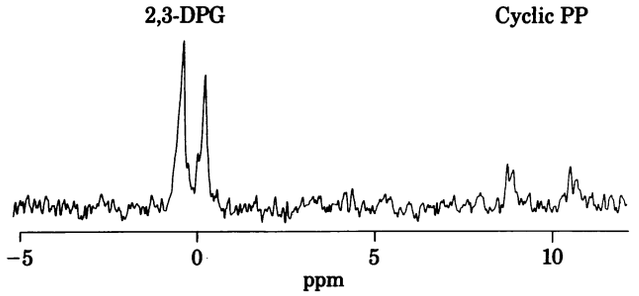


FIG. 6. $Ba(OH)_2$ degradation of the cyclic pyrophosphate: The sample was incubated with $Ba(OH)_2$ as described, and then the pH was adjusted to 3.5 (to solubilize all components). The doublet around 0 ppm was positively identified as 2,3-diphosphoglycerate (2,3-DPG) by spiking with authentic material.

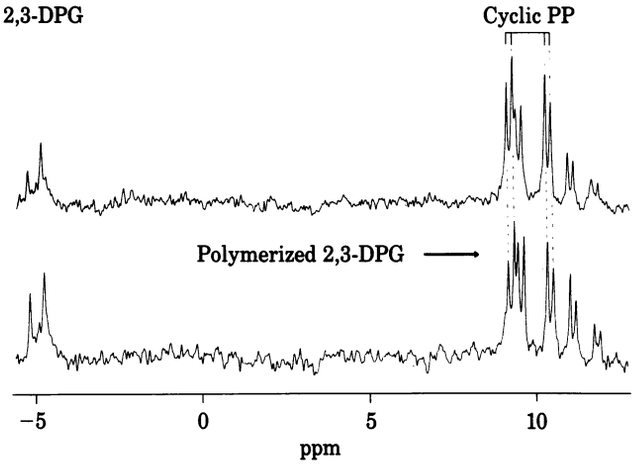


FIG. 7. Cyclization of dilute 2,3-diphosphoglycerate (2,3-DPG) with dicyclohexylcarbodiimide. (*Upper*) ^{31}P spectrum of products at pH 9.7 (after Dowex H^+ exchange and neutralization with NaOH). (*Lower*) Same sample with a small amount of cyclic pyrophosphate added. The dashed lines identify the cyclic pyrophosphate AB quartet.

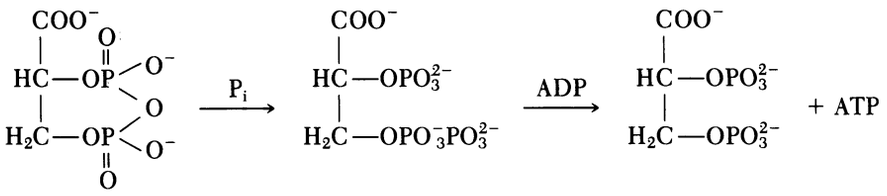
m/z of 293. An exact mass measurement of this ion (by using the glycerol cluster ion of m/z = 277 as the reference) was 292.9201, which agrees well with the predicted mass for the molecular disodium-cationized ion $(C_3H_5O_9Na_2)^+$ of 292.9204. This corresponds to the disodium salt of structure A—i.e., the disodium salt of 2,3-cyclopyrophosphoglyceric acid.

Selective Degradation of the Pyrophosphate. $Ba(OH)_2$ is known to hydrolyze pyrophosphate linkages without affecting monophosphate esters (19). A solution of the isolated pyrophosphate (10 mM) was treated with an excess of solid $Ba(OH)_2$ and heated at 40°C for 30 min. The colloidal solution was decanted from excess $Ba(OH)_2$, mixed with small amounts of H_2O, then passed over Dowex H^+, and neutralized to pH 4.5 with NaOH. ^{31}P NMR spectra of the product showed a breakdown of the novel pyrophosphate compound to yield compounds with ^{31}P resonances in the phosphate monoester region (Fig. 6). The major hydrolyzed product was identified as 2,3-diphosphoglycerate by spiking the hydrolysate with authentic material.

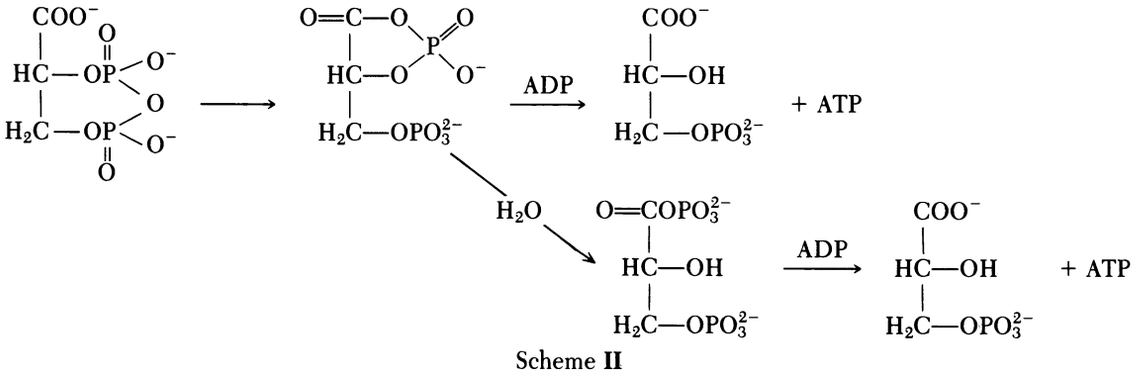
Synthetic Cyclization of 2,3-Diphosphoglycerate. Because this unique pyrophosphate can be degraded to 2,3-diphosphoglycerate, it also should be synthesized (albeit perhaps as a minor product) by intramolecular cyclization of that compound under dilute conditions in the presence of dicyclohexylcarbodiimide (20, 21). The pyridinium salt of 2,3-diphosphoglycerate (10 mM) was incubated in anhydrous pyridine/tributylamine with 100 mM dicyclohexylcarbodiimide at room temperature for 30 hr. ^{31}P NMR spectra of the reaction products showed the presence of various pyrophosphate compounds (Fig. 7). Upon addition of the cyclic pyrophosphate, a set of four resonances increased in intensity. Thus, under these conditions, the major product of the cyclization of 2,3-diphosphoglycerate is the same cyclic pyrophosphate that was isolated from methanogens.

DISCUSSION

Methanogens form a unique class of anaerobes capable of reducing CO_2 to methane. *In vivo* ^{31}P NMR experiments have shown that they possess a high concentration (roughly 10–12 mM) of a novel pyrophosphate compound that has been purified and identified. This compound was not observed or postulated in any other studies of methanogens, and its detection illustrates the utility of *in vivo* ^{31}P NMR. This cyclic pyrophosphate structure can be degraded specifically to 2,3-diphosphoglycerate and synthesized as one of many products of dicyclohexylcarbodiimide cyclization of 2,3-diphosphoglycerate. The major question is what is the role of this pyrophosphate compound in *M. thermoautotrophicum* metabolism? Several possibilities exist: 2,3-cyclopyrophosphoglycerate is (*i*) a storage compound for phosphate (much like polyphosphates in other bacteria), (*ii*) an allosteric effector of some critical enzyme (similar to 2,3-diphosphoglycerate regulation of hemoglobin in red blood cells), or (*iii*) a novel phosphagen in the energy metabolism of these archaebacteria. As a phosphagen, the cyclic pyrophosphate would have a role similar to creatine phosphate in muscle, where the latter compound (≈20 mM) provides a reserve of high-energy phosphoryl groups and keeps the adenylate system of muscle buffered at high "energy charge." Two such series of phosphate transfer reactions (mediated by enzymes) from cyclic pyrophosphoglycerate to 2,3-diphosphoglycerate or 3-phosphoglycerate (or 2-phosphoglycerate) could be operational in these methanogens:



Scheme I



Scheme II

Intracellular ATP has been estimated in another methanogen strain (*Methanobacterium* strain MOH) as 1.7 mM (22), with a comparable value of ADP. The ATP system must be in kinetic equilibrium between ATP-producing and ATP-consuming reactions. In scheme **I** a modified 2,3-diphosphoglycerate could be the form that phosphorylates ADP. This intermediate is somewhat reminiscent of 3-adenosine diphosphate phosphoglycerate, an unusual derivative found in mammalian blood (23). If the overall scheme is correct, ^{31}P resonances for the cyclic pyrophosphate, 2,3-diphosphoglycerate, and the intermediate should be visible in extracts.

In scheme **II** a cyclic acyl phosphate is formed that could directly phosphorylate ADP or hydrolyze to form 1,3-diphosphoglycerate, a glycolytic intermediate linked to ADP phosphorylation. This latter scheme would predict the presence of 3-phosphoglycerate (or 2-phosphoglycerate produced through the appropriate mutase) in extracts. The sugar phosphate region of Fig. 2 can be further resolved (by spiking extracts with authentic material) into fructose 6-phosphate, 2,3-diphosphoglycerate, possibly 2-phosphoglycerate, but not 3-phosphoglycerate. A small resonance around 6 ppm in the terminal phosphate region of monoester pyrophosphates is not ADP or free pyrophosphate. Together with the resonance between the cyclic pyrophosphate AB quartet peaks and the unidentified resonance in the sugar phosphate region, it may reflect the intermediate postulated in the interconversion scheme **I**. While these extracts are consistent with such ATP-regulation schemes, further evidence will require isolation of intermediates and isolation of the methanogen enzymatic activities responsible for these interconversions.

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