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# Cyclic 2,3-Diphosphoglycerate as a Component of a New Branch in Gluconeogenesis in *Methanobacterium thermoautotrophicum* ΔH

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**A unique compound, cyclic 2,3-diphosphoglycerate (cDPG), is the major soluble carbon and phosphorus solute in *Methanobacterium thermoautotrophicum* ΔH under optimal conditions of cell growth. It is a component of an unusual branch in gluconeogenesis in these bacteria. [U-$^{13}$C]acetate pulse-[$^{12}$C]acetate chase methodology was used to observe the relationship between cDPG and other metabolites (2-phosphoglycerate and 2,3-diphosphoglycerate [2-PG and 2,3-DPG, respectively]) of this branch. It was demonstrated that cells could grow exponentially under conditions in which 2-PG and 2,3-DPG, rather than cDPG, were the major solutes. While the total concentration of these three phosphorylated molecules was maintained, rapid interconversion of $^{13}$C label among them was observed. Label flow from 2-PG to 2,3-DPG to cDPG to polymer is the usual direction in this pathway in exponentially growing cells, while the reverse reactions sometimes predominate in the stationary phase. Evidence of the presence of a polymeric compound in this pathway was provided by $^{13}$C nuclear magnetic resonance (one-dimensional and two-dimensional INADEQUATE) studies of solubilized cell debris.**

Methanogens are a very diverse group of the class *Archaeobacteria* able to generate energy by reducing a variety of substrates, including formate, acetate, methanol, mono-, di-, and trimethylamine, CO, and $CO_2$, to methane (1, 3, 10, 13, 24). A variety of unusual and unique compounds have been found in methanogens; these include coenzyme M (17), methanofuran (16), and methanopterin (12), which are essential for methanogenesis. Another unique compound, cyclic 2,3-diphosphoglycerate (cDPG) (11, 20), is found at moderate to high concentrations in a subset of methanogens including two major phylogenetic groups: *Methanobacteriales* (*Methanobacterium thermoautotrophicum*, *Methanobrevibacter smithii*, and *Methanothermus fervidus*) and *Methanomicrobiales* (*Methanosarcina frisia*) (9, 19, 21). cDPG has also been detected at low concentrations in *Methanosphaera stadtmanae* (19). cDPG is the major soluble carbon and phosphorus solute in *M. thermoautotrophicum* ΔH. A connection between cDPG formation and degradation and gluconeogenesis has been proposed (4); however, there are incomplete in vivo data on the relationship and interconversion of cDPG with the usual phosphorylated $C_3$ intermediates of this pathway. In vivo and in vitro nuclear magnetic resonance (NMR) studies of *M. thermoautotrophicum* ΔH have shown that under certain conditions (e.g., lowered temperature), 2,3-diphosphoglycerate (2,3-DPG) was the product of the enzymatic degradation of cDPG (the enzyme responsible, cDPGase, has been purified and characterized) (19). Under those conditions, the synthesis of cDPG from 2,3-DPG was not observed. Other in vitro work, with *M. fervidus*, showed that enzyme extracts could form cDPG from 2,3-DPG and ATP, but the degradation of cDPG back to 2,3-DPG was not observed (15). In vivo studies that reconcile these two observations are necessary to understand the role of the unusual cyclic $PP_i$ compound in methanogens. It is this relationship that this work addresses.

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## MATERIALS AND METHODS

**Chemicals.** $CO_2$-$H_2$ (1:4 [vol/vol]) and $CO_2$-$N_2$ (1:4 [vol/vol]) were acquired from WESCO Co. [U-$^{13}$C]acetate (99% pure) was acquired from Cambridge Isotope. Other medium components were of reagent grade. Dimethyl sulfoxide (DMSO)-$^2H_6$ was acquired from Merck Sharp and Dohme International. $^2H_2O$ and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co.

**$^{13}$C labeling of cellular material.** Cells of *M. thermoautotrophicum* ΔH were grown with the media and conditions described previously (19). Liquid medium (150 ml) in 500-ml

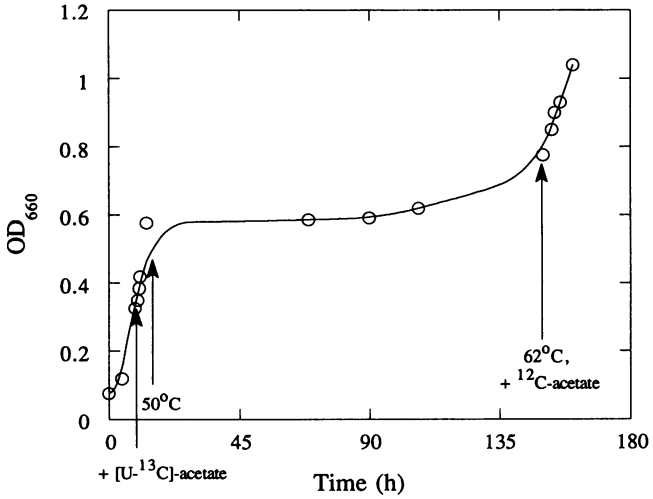


FIG. 1. $A_{660}$ as a function of incubation time for *M. thermoautotrophicum* ΔH growing at 62°C in the presence of 5 mM [U-$^{13}$C] acetate from the point marked by the first arrow, incubated at 50°C (second arrow), and subsequently diluted with [$^{12}$C]acetate (to a final concentration of 50 mM) and incubated at 62°C (third arrow). $OD_{660}$, optical density at 660 nm.

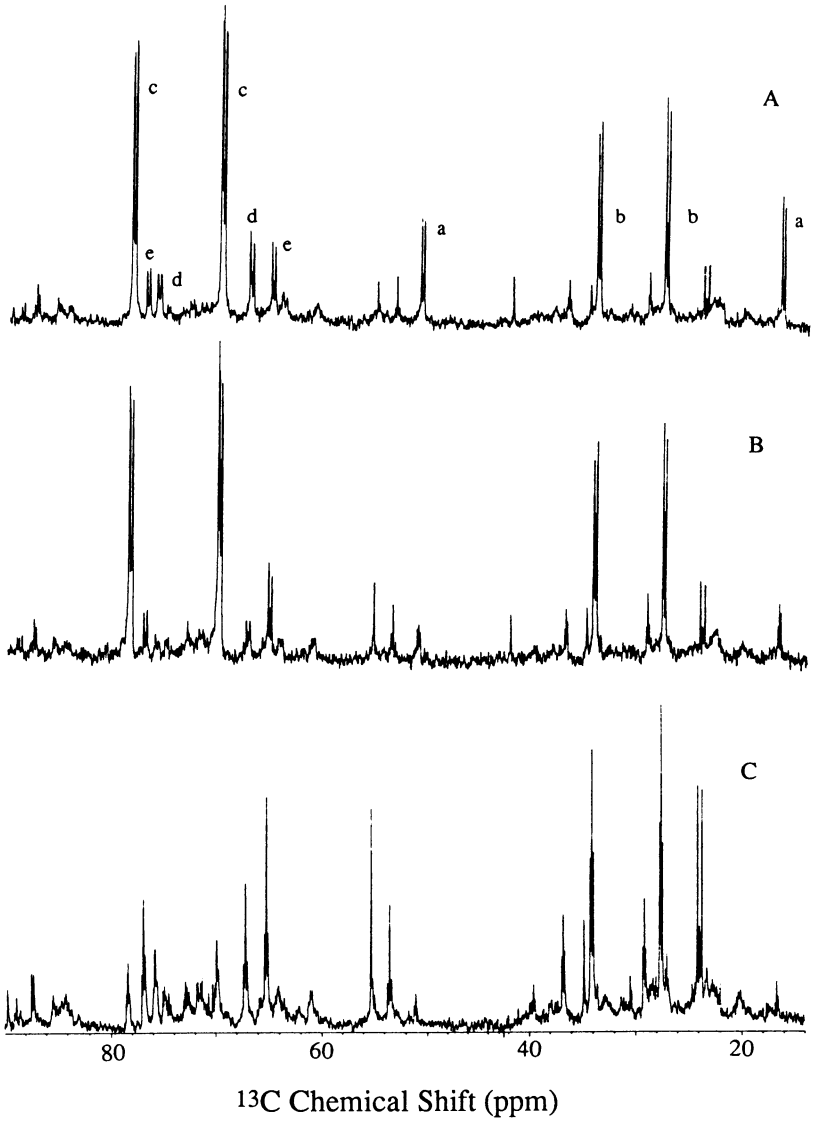


FIG. 2. $^{13}C$ (125.7-MHz) NMR spectra of ethanol extracts of the *M. thermoautotrophicum* cells shown in Fig. 1 0 (A), 1 (B), and 8 (C) h after dilution with [$^{12}C$]acetate and resumption of growth at 62°C. $^{13}C$ resonances belonging to key compounds are labeled as follows: a, alanine; b, glutamate; c, cDPG; d, 2,3-DPG; e, 2-PG.

pressurized bottles (in a typical experiment, six to eight bottles were used) was inoculated with 10 ml of fresh inoculum (e.g., cells grown in medium to an $A_{660}$ of 0.6 and then used immediately for inoculation). Cells were grown in a shaker bath at 62°C, with venting and repressurizing with $CO_2$-$H_2$ (20 lb/in$^2$) every 4 h until the cell suspension reached an $A_{660}$ of ~0.4. For the [U-$^{13}C$]acetate pulse-[$^{12}C$]acetate chase experiment, 1.0 ml of 0.71 M [U-$^{13}C$]acetate solution was added anaerobically (to a final concentration of 5 mM), and growth was allowed to proceed to an $A_{660}$ of ~0.55. For induction of the cells to accumulate NMR-detectable amounts of 2,3-DPG and 2-phosphoglycesate (2-PG), cells were subsequently incubated at 50°C for 30 to 90 h. The agitation speed and gas composition ($CO_2$-$H_2$ [1:4, vol/vol] or $CO_2$-$H_2$-$N_2$ [1:2:2, vol/vol/vol]) were varied to keep the cells viable and able to grow exponentially (or to maintain them in the stationary phase) in the [$^{12}C$]acetate chase part of the experiment. During the incubation period, the pool of phosphorus-containing molecules was analyzed in vivo and in vitro (ethanol extracts) by $^{31}P$ NMR spectroscopy. Five milliliters of medium from each bottle was used (in combination) to prepare one sample for spectral analysis. Finally, 3 ml of 2.55 M stock [$^{12}C$]acetate solution (producing a final concentration of 50 mM) was added to each bottle, and growth was allowed to proceed at 62°C. Cells were harvested at various times after the start of the chase.

**Ethanol extraction.** Harvested cells were transferred to centrifuge bottles and centrifuged in a Beckman JA-10 rotor at 10,000 × *g* and 4°C for 30 min. The supernatant was discarded, leaving ~1.0 to 1.5 ml of residual medium. The cells were resuspended, transferred to Eppendorf Microfuge tubes, and centrifuged in the Microfuge at 10,000 × *g* for 10 min. The supernatant was discarded, and the cell pellet was extracted three times with 1 ml of 70% ethanol as described previously (19). The solvent from the combined supernatants was removed under vacuum, and the residue was dissolved

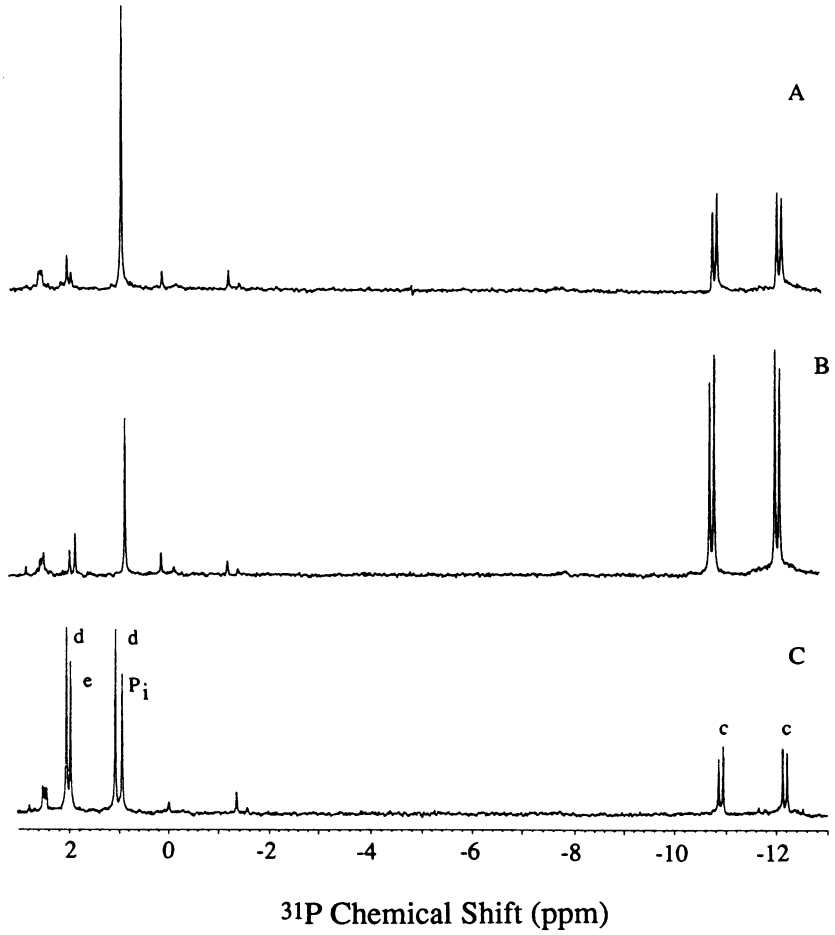


FIG. 3. $^{31}P$ (202.3-MHz) NMR spectra of the ethanol extracts of the *M. thermoautotrophicum* cells shown in Fig. 2 0 (A), 1 (B), and 8 (C) h after dilution with [$^{12}C$]acetate and resumption of growth at 62°C. $^{31}P$ resonances are identified as belonging to cDPG (c), 2,3-DPG (d), and 2-PG (e).

in 1.5 ml of $H_2O$. After the addition of 1 drop of 1 M HCl, the sample was vortexed, frozen, and lyophilized overnight. A typical yield from 150 ml of medium at an $A_{660}$ of 0.78 was 15.4 mg of ethanol-extractable material, 27% of the cell dry weight. Ten to 20 mg of the ethanol extract was dissolved in 500 μl of $^2H_2O$, centrifuged at 10,000 × *g* if cloudy, and transferred to a 5-mm NMR tube for analysis by $^{13}C$ NMR. For the acquisition of a $^{31}P$ spectrum, 50 μl of 10 mM EDTA was added, and the pH was adjusted to 7.0. The molar concentrations of phosphorylated $C_3$ species in the ethanol extract were determined by colorimetric quantitation of inorganic phosphate in the sample after $HClO_4$ digestion (22) by use of the integrated $^{31}P$ intensity of the species and based on the assumption that the cell volume was 2.5 μl/mg of cell dry weight.

**Natural-abundance specific $^{13}C$ intensity measurement and error estimation.** Three samples of *M. thermoautotrophicum* ethanol extracts (each from 150 ml of standard medium grown to an $A_{660}$ of ~1) were treated with 2,3-DPG to yield approximately 0.3, 0.5, and 0.7 the normal cDPG level. The standard deviation for integrated $^{13}C$ intensity normalized to integrated $^{31}P$ intensity for the C-2 atom of cDPG and 2,3-DPG was calculated to be 10.4% (mean value, 0.095; integrated $^{13}C$ and $^{31}P$ scales equal to 10,000 and 350,000, respectively; 12 measurements). This result indicates that the measurement of intensities by NMR is accurate to ±10% when carried out in this fashion. Hence, comparisons of enrichment levels, the main focus here, are quite accurate.

**Preparation of cell debris for NMR analysis.** The cell debris (after ethanol extraction) was resuspended in 1.0 ml of $H_2O$, sonicated for 3 min, vortexed after the addition of 1 drop of 1 M HCl, frozen, and lyophilized overnight. This cell suspension would yield 41.2 mg of material (73% of the cell dry weight). The lyophilized powder (30 to 50 mg) was suspended in 600 to 700 μl of DMSO-$^2H_6$, and 30 to 35 μl of TFA was added. The suspension was vortexed for 3 min and bath sonicated (Branson model C4-6) at 50°C for 10 min. This operation was repeated two to five times to yield a viscous yellow to greenish solution that was transferred to a 5-mm NMR tube.

**NMR spectroscopy.** WALTZ $^1H$-decoupled $^{13}C$ NMR spectra (125.7 MHz) of ethanol extracts were acquired on a Varian Unity 500 spectrometer with a 25-kHz sweep width, 32,768 datum points, a 6.5-μs pulse width (45°), a 0.64-s acquisition time, 4.0-Hz line broadening, and 2,000 transients. $^{13}C$ chemical shifts were referenced to the internal ethanol $CH_3$ at 17.2 ppm. WALTZ $^1H$-decoupled $^{31}P$ NMR spectra (202.3 MHz) were obtained on the same spectrometer with a 6-kHz sweep width, 16,384 datum points, a 9.3-μs pulse width, a 0.779-s acquisition time, 2.0-Hz line broaden-

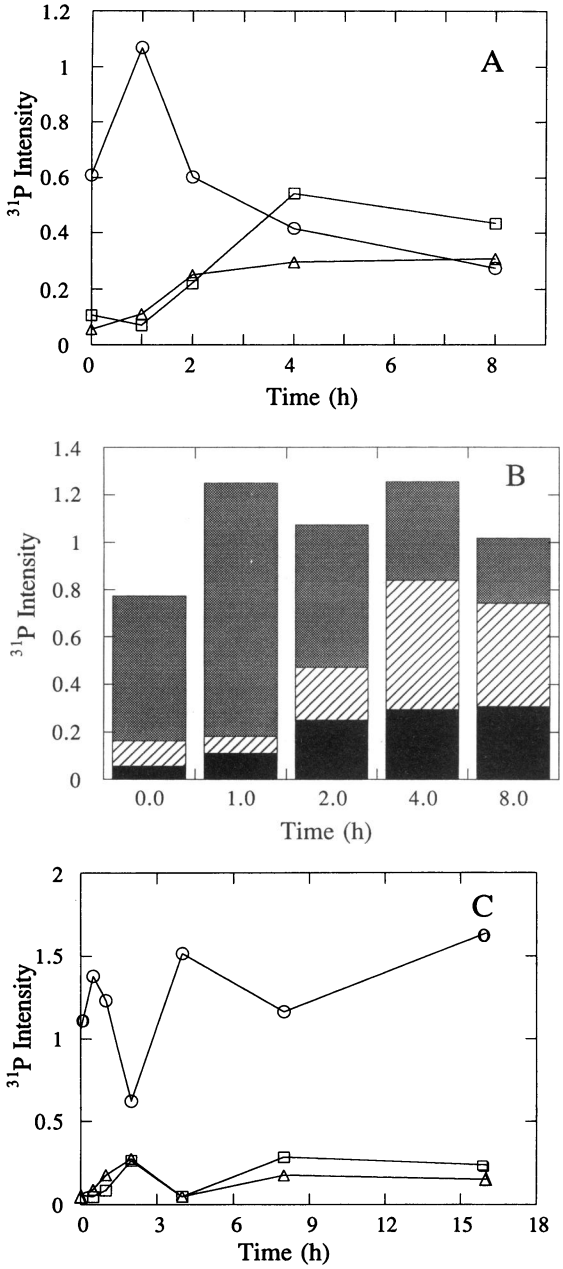


FIG. 4. (A) Integrated $^{31}$P intensity of cDPG (○), 2,3-DPG (□), and 2-PG (△) as a function of time during the chase phase of the [U-$^{13}$C]acetate pulse-[U-$^{12}$C]acetate chase experiment with exponentially (at 62°C) growing cells. Growth resumed, after incubation at 50°C for 100 h, with the addition of [$^{12}$C]acetate and incubation at 62°C. (B) Total integrated $^{31}$P intensity of cDPG (shaded bars), 2,3-DPG (hatched bars), and 2-PG (black bars) during the chase phase. (C) A different experiment showing the integrated $^{31}$P intensity of 2-PG, 2,3-DPG, and cDPG after incubation at 50°C for 50 h and resumption of growth at 62°C (same symbols for each compound as in panel A).

ing, and 200 transients. $^{31}$P chemical shifts were referenced to an external capillary of $H_3PO_4$. Under these conditions, neither $^{13}$C nor $^{31}$P nuclei are saturated. Furthermore, all CH, all $CH_2$, and all phosphate esters have similar nuclear Overhauser enhancements. Therefore, direct comparisons of integrated intensity reflect concentrations of nuclear label.

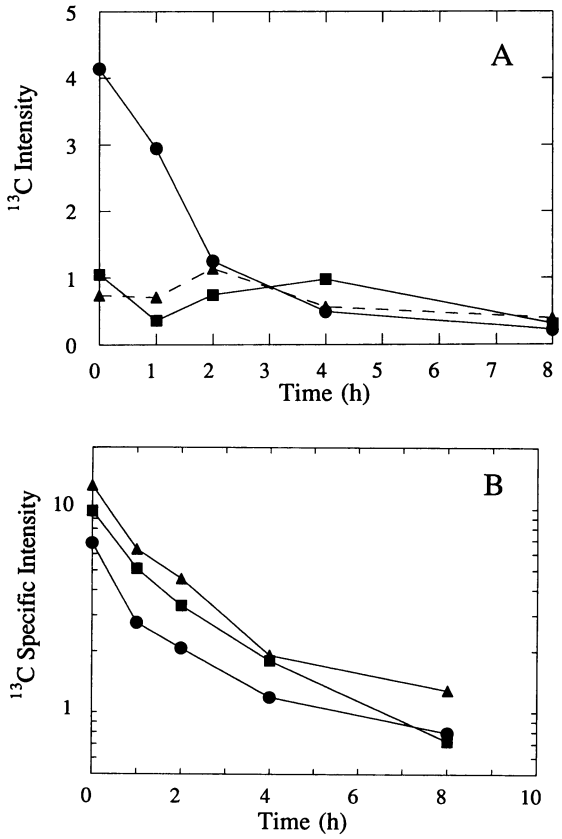


FIG. 5. (A) Integrated $^{13}$C intensity of $CH_2$ groups of cDPG (●), 2,3-DPG (■), and 2-PG (▲) during the chase phase of a [U-$^{13}$C] acetate pulse-[U-$^{12}$C]acetate chase experiment with exponentially growing cells. Growth resumed after incubation at 50°C for 100 h and dilution of the $^{13}$C label with [$^{12}$C]acetate. (B) Specific $^{13}$C intensity (i.e., normalized to $^{31}$P content) of cDPG, 2,3-DPG, and 2-PG as a function of time during the chase; symbols are as in panel A.

For obtaining $^{13}$C NMR spectra of solubilized cell debris, half as many datum points (since the resonances were considerably broader) and a slightly longer pulse width (8.0 μs) were used. Typically, 15,000 to 30,000 transients were collected, and the data were processed with 10-Hz line broadening.

For observation of only doubly ($^{13}$C-$^{13}$C) labeled species in the small molecule pool (ethanol extracts) or in the cell polymeric material (debris), $^{13}$C NMR spectra were obtained in a $^{13}$C NMR (one-dimensional and two-dimensional [1D and 2D, respectively]) INADEQUATE experiment (2). For

TABLE 1. Half-life of $^{13}$C turnover in phosphorylated $C_3$ molecules in *M. thermoautotrophicum* ΔH[a]

| Solute | Half-life (h) | |
|---|---|---|
| | Initial[b] | Subsequent[c] |
| cDPG | 0.3 | 1.7 |
| 2,3-DPG | 0.4 | 1.1 |
| 2-PG | 0.4 | 1.3 |

[a] Cells were grown exponentially at 62°C.
[b] Evaluated for the first hour of the $^{12}$C chase.
[c] Evaluated for 1 to 7 h after the start of the $^{12}$C chase.

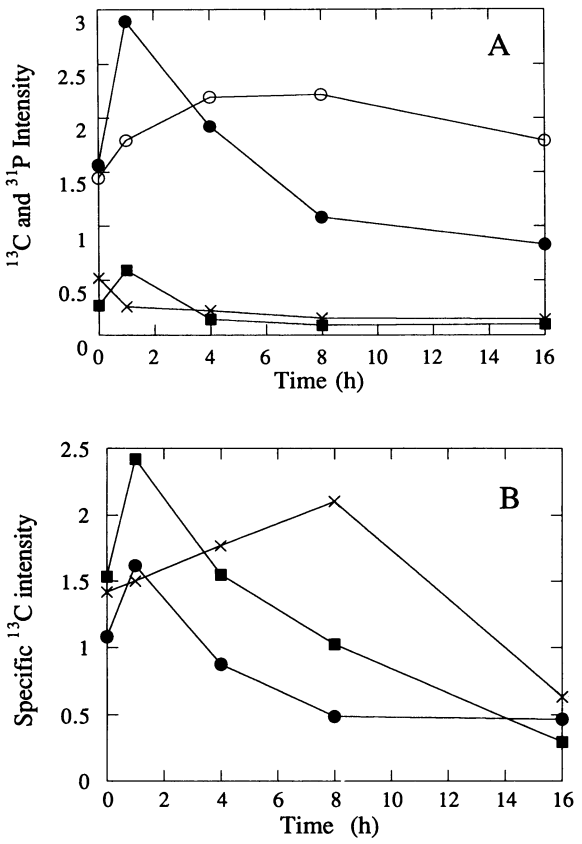


FIG. 6. (A) Integrated $^{13}$C intensity of cDPG (●), 2,3-DPG (■), and alanine (×) and integrated $^{31}$P intensity of cDPG (○) as a function of time during the chase phase of a [U-$^{13}$C]acetate pulse-[U-$^{12}$C]acetate chase experiment with cells that were grown to the stationary phase and incubated at 50°C in the presence of 5 mM [U-$^{13}$C]acetate. (B) Specific $^{13}$C intensity (normalized to $^{31}$P content) of cDPG (●) and 2,3-DPG (■) as a function of time after the start of the chase. The 2,3-DPG/cDPG specific $^{13}$C intensity ratio is also indicated (×).

obtaining 2D INADEQUATE spectra, 250 mg of dry ethanol extract was dissolved in 500 μl of $D_2O$. A data matrix ranging from 185 to 10 ppm consisted of 192 (F1 dimension) by 2,000 (F2 dimension) points; the spectral width for F1 was 45,584 Hz, and that for F2 was 22,972 Hz.

## RESULTS

[$^{13}C_2$]acetate pulse-[$^{12}$C]acetate chase methodology in combination with $^{31}$P and $^{13}$C NMR spectroscopy is a convenient way to analyze the dynamics of cDPG turnover in *M. thermoautotrophicum* ΔH, since acetate is readily incorporated into small molecule pools (5, 6). From the early days of its discovery in *M. thermoautotrophicum* ΔH, there was a tendency to connect cDPG availability in this bacterium to carbon and phosphorus storage for gluconeogenesis and carbohydrate synthesis. In *M. thermoautotrophicum* under normal growth conditions, phosphoenolpyruvate (PEP), 2-PG, 3-phosphoglycerate (3-PG), 2,3-DPG, and 1,3-diphosphoglycerate are not available in NMR-detectable amounts for direct observation of the relationship among those species. Recently (19), it was shown that intact cells can be induced to accumulate substantial amounts of 2,3-DPG by lowering of the temperature from 62 to 50°C. Moreover, studies in vitro at 37°C (and in vivo at 50°C) have shown that 2,3-DPG is the product of the enzymatic degradation of cDPG under these conditions. To connect this reaction with other metabolites, we induced the cells to accumulate 2,3-DPG and 2-PG in amounts sufficient for NMR studies. Variations in cell incubation temperature, gas mixture composition, and intensity of agitation during the growth of *M. thermoautotrophicum* were used to alter the levels of other phosphorylated metabolites.

Under no conditions was 3-PG observed at NMR-detectable levels. Even when the bacteria were growing in the presence of 150 mM 3-PG in the medium, only trace amounts of 3-PG could be observed in cell extracts by $^{31}$P NMR. In contrast, the concentration of 2-PG in this methanogen could be controlled by varying the external conditions. After incubation at 50°C for 50 to more than 100 h in an atmosphere gradually shifted from $CO_2$-$H_2$ (1:4 [vol/vol]) to $CO_2$-$H_2$-$N_2$ (1:2:2 [vol/vol/vol]), cells were still able to resume exponential growth at 62°C (Fig. 1), with a doubling time of 9.4 to 10.0 h. Under these conditions, the composition of the phosphorylated small molecule pool was very unusual during the first 8 h after resumption of growth (Fig. 2 and 3). For cells incubated at 50°C for 100 h, instead of the cDPG concentration being maintained at the same high stable level as that observed under normal growth conditions, the total concentration of the three phosphorylated molecules (cDPG plus 2,3-DPG plus 2-PG) was maintained (Fig. 4A and B), and the rapid interconversion of $^{13}$C label among these three species was observed. The concentration of internal $P_i$ was also correlated with the mutual interconversion of phosphorylated three-carbon molecules. In the first hour after a return to 62°C, a rapid increase in the cDPG concentration was accompanied by a decrease in the $P_i$ concentration. At 2 to 4 h after the resumption of exponential growth, the cells showed a decreased cDPG level and increased $P_i$, 2-PG, and 2,3-DPG levels. The half-life for $^{13}$C label in all these molecules, as measured from $^{13}$C turnover, appeared to be very short in comparison with the cell doubling time.

The relative concentrations of 2,3-DPG, 2-PG, and cDPG were determined by measurement of integrated intensities in $^{31}$P NMR spectra at 1.8, 1.7, and −10.9 ppm, respectively (for comparison of the phosphorus atom attached to C-2 in each of these molecules). At the beginning of growth in this experiment, the 2,3-DPG and 2-PG levels were 18% (4.2 mM) and 9% (2.3 mM) the cDPG (24.4 mM) level, while at the end of the eighth hour, they were 154% (16.9 mM) and 112% (12.3 mM) the cDPG (11.0 mM) level (Fig. 4A). In a different experiment (50-h incubation time at 50°C, no feeding or changes in gas composition), at least three different cycles of phosphorylated small molecule interconversion could be distinguished. The degradation of cDPG (after some initial growth in the first 0.5 h at 62°C) in the time period from 0.5 to 2 h at 62°C was accompanied by increases in 2,3-DPG and 2-PG levels. After 2 h, decreasing 2,3-DPG and 2-PG levels were correlated with an increasing cDPG level (Fig. 4C). The correlation of the change in the levels of 2,3-DPG, 2-PG, and cDPG on a short time scale is evident (Fig. 4A and C). Slight variations of these experiments were repeated three other times, with similar results. Thus, in vivo the degradation of cDPG was correlated with an increased 2,3-DPG level and, to a lesser extent, an increased 2-PG level, but the same was true in the reverse direction. When the cDPG level increased, those of the other two molecules decreased.

Taking into account the rapid interconversion among

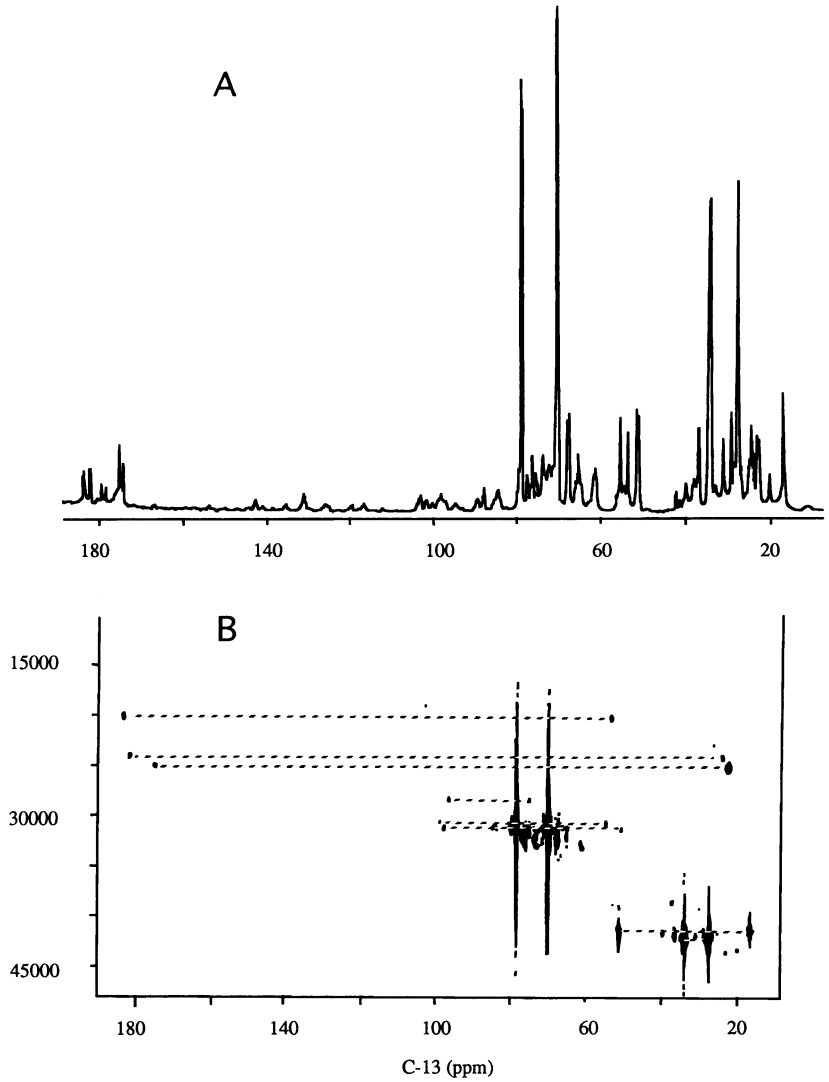


FIG. 7. $^{13}C$ INADEQUATE (125.7-MHz) NMR spectrum (10 to 190 ppm) of ethanol extracts of *M. thermoautotrophicum* grown on [U-$^{13}C$]acetate. Broken lines connect $^{13}C$-$^{13}C$-coupled signals. (A) 1-D $^{13}C$ spectrum; (B) 2-D contour plot.

these species, we can assess the direction of label flow by measuring specific $^{13}C$ intensities of similar carbon atoms (C-3 is more convenient for this purpose because it is not affected by the partial breakdown of [U-$^{13}C$]acetate and the reformation of [2-$^{13}C$]acetate [6]). $^{13}C$ natural abundance is 1.1%. The dilution of [U-$^{13}C$]acetate in the medium (5 mM) with [$^{12}C$]acetate (50 mM) in these experiments should eventually lower the specific $^{13}C$ content of the species of interest to the new equilibrium level, approximately 1/10 the original one. This is indeed what was observed. Integrated $^{13}C$ intensity (Fig. 5A) normalized to integrated $^{31}P$ intensity monitored the specific $^{13}C$ intensities of the phosphorylated molecules as a function of time (Fig. 5B). The kinetics of $^{13}C$ label turnover were not first order, as is clearly shown in the plot of log $^{13}C$ versus time (Fig. 5B). During the first hour of cell growth after return of the temperature to 62°C, $^{13}C$ label content decreased more rapidly than at points after 1 h. The nonlinear curve in Fig. 5B can be treated as the sum of two exponentials. The half-life for label turnover within the first hour was extrapolated to be 30 to 40 min; for turnover during the subsequent 7 h, the half-life was calculated to be 1.1 to 1.7 h (Table 1). In any event, the $^{13}C$ label turnover was much more rapid than cell growth, as represented by the doubling time. In Fig. 5B, the specific $^{13}C$ content decreased from 2-PG to 2,3-DPG to cDPG, and this tendency was conserved throughout the time scale of the experiment. As label was diluted among these compounds, eventually all three should have had the same specific $^{13}C$ content, yet they clearly did not. Furthermore, given the accuracy in measuring $^{13}C$ content, which is ~10% in the $^{13}C$-$^{31}P$ NMR experiments, the differences in the specific $^{13}C$ contents of the three phosphorylated species were quite significant. The observed results are consistent with the direction of label flow from 2-PG to 2,3-DPG to cDPG, but with cDPG also rapidly interconverting with some polymeric material (there is no good candidate in the observable small molecule pool) labeled less than 10% with $^{13}C$. If this suggestion is true, it should be possible to observe the net flow of $^{13}C$ label back into the small molecule pool when catabolic reactions predominate over anabolic ones.

For approximation of this situation, cells were grown to the stationary phase in the presence of [U-$^{13}C$]acetate (incu-

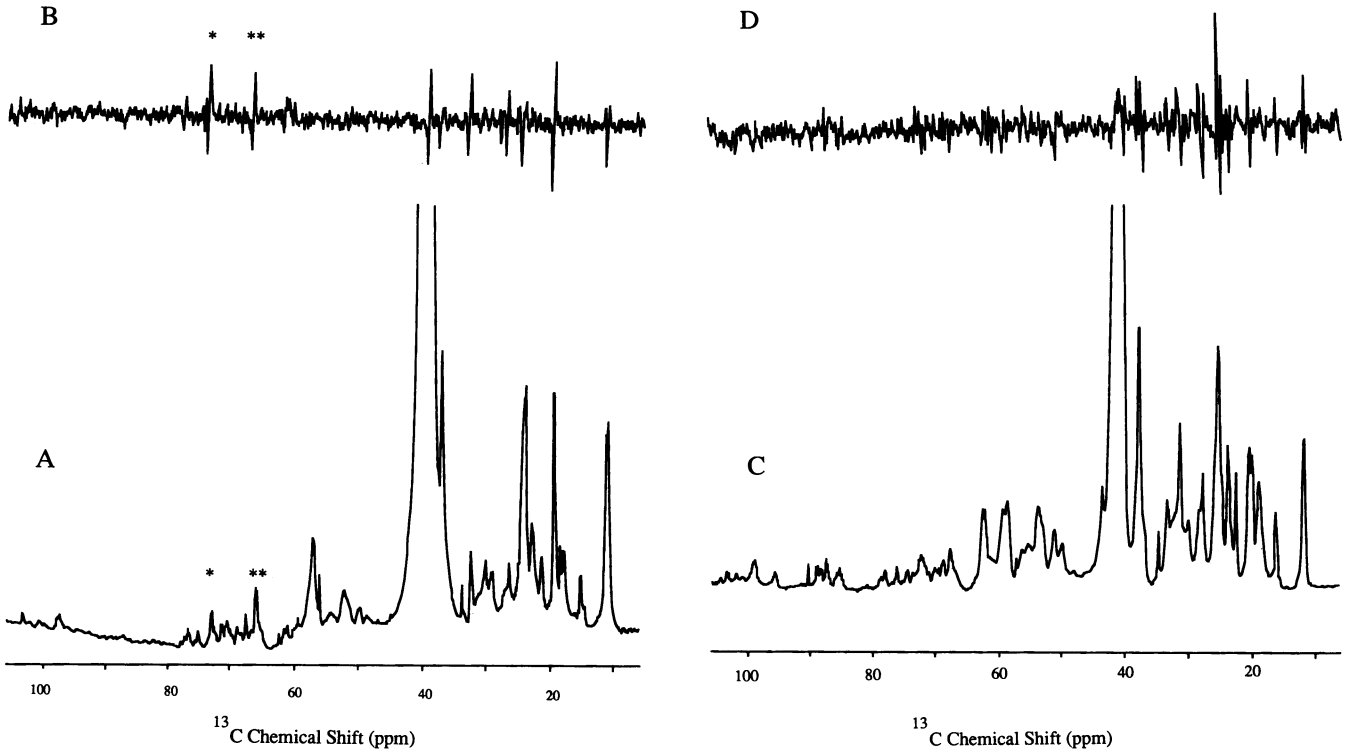


FIG. 8. (A and C) $^{13}C$ and (B and D) $^{13}C$ (1D) INADEQUATE (125.7-MHz) NMR spectra of intact cell debris solubilized in DMSO-$^{2}H_6$ plus TFA for *M. thermoautotrophicum* grown on [U-$^{13}C$]acetate at 62°C, incubated at 50°C, diluted with [U-$^{12}C$]acetate, and grown again at 62°C. Panels A and B and panels C and D show data obtained 0 h and 8 h, respectively, after the resumption of growth at 62°C. The resonances marked with asterisks and double asterisks represent carbons whose chemical shifts were at 74 and 67 ppm, respectively.

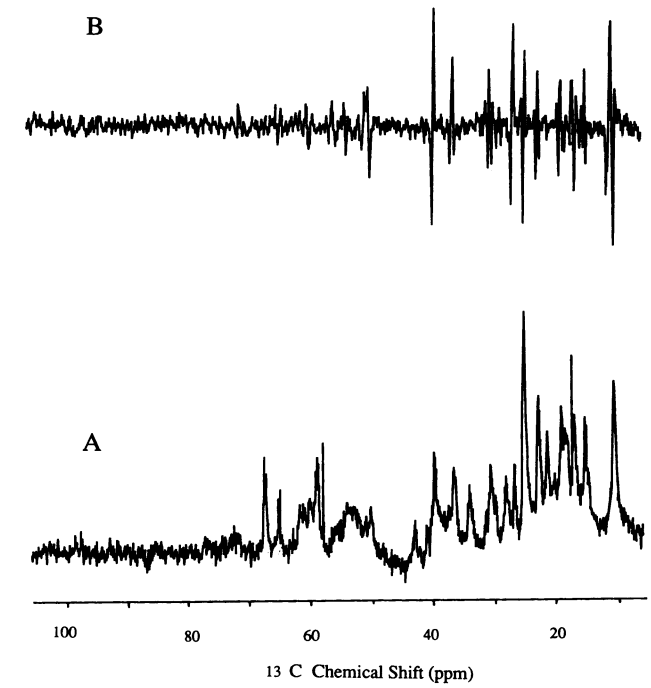


FIG. 9. (A) $^{13}C$ and (B) $^{13}C$ (1D) INADEQUATE (125.7-MHz) NMR spectra of water extracts (obtained after preliminary ethanol extraction) of *M. thermoautotrophicum* grown on [U-$^{13}C$]acetate at 62°C.

bated initially at 62°C and then at 50°C from an $A_{660}$ of 0.36 to an $A_{660}$ of 0.95). This treatment would be expected to enrich the postulated polymeric recipient of the label to the extent that the label backflow could actually be seen. Indeed (Fig. 6A), during the first hour of return of the cells to 62°C, an initial increase in the specific $^{13}C$ content was observed for both cDPG and 2,3-DPG. For comparison, alanine, which is normally the first molecule to be labeled by [U-$^{13}C$]acetate because it is directly synthesized from pyruvate, exhibited a rapid loss of $^{13}C$ label. In these experiments, the amounts of 2-PG produced were not sufficient to include this molecule in the analysis.

This increase in $^{13}C$ label content in cDPG cannot be explained by the simultaneous increase in the cDPG concentration, as reflected by integrated $^{31}P$ intensity measurements (Fig. 6A). Initially, the 2,3-DPG pool was more enriched than the cDPG pool, and this result was conserved in the first 8 h, with the relative $^{13}C$ label flow in the 2,3-DPG direction. It is interesting that despite the complex character of label flow, this dependence was linear (Fig. 6B).

What molecule is the final recipient of the $^{13}C$ label from the cDPG pool? Or, if there are many, is there one in particular that is responsible for returning the largely intact $^{13}C$-$^{13}C$ label back to the small molecule pool under certain conditions? There is evidence that carbohydrate metabolism is unidirectional in *M. thermoautotrophicum* (8). On the other hand, glycogen has been reported to occur in a few *Methanococcales* and *Methanomicrobiales* species (14, 18). The possibility of a functional glycolysis pathway related to cDPG can be checked by $^{13}C$ NMR spectroscopy. In cDPG synthesis, the carbonyl atom comes from $CO_2$ rather than

from [$^{13}C_2$]acetate and is not labeled (6). In the reverse pathway, from glucose, the action of fructose 1,6-bisphosphate aldolase and triose phosphate isomerase would label the cDPG carbonyl atom with $^{13}C$ to some extent. The data obtained from $^{13}C$ (2D) INADEQUATE NMR studies of cell extracts (Fig. 7) revealed no correlation between signals in the carbonyl region and the CH (79 ppm) atom of cDPG. The three carbonyl atoms that enter into double-quantum coherence have chemical shifts at 184, 182.5, and 176 ppm and correlate with signals at 54, 25, and 23 ppm, respectively. Therefore, even if carbohydrate degradation actually took place to some extent, it did not contribute significantly to the observable $^{13}C$ label flow back into cDPG.

The $^{13}C$ NMR studies of the cell polymeric material provided information consistent with these data. $^{13}C$ NMR spectra of the intact cell debris (after ethanol extraction) were obtained with DMSO-$^2H_6$ and TFA as a solvent system. These fairly complicated spectra (Fig. 8A) could be dramatically simplified by measuring only the $^{13}C$-$^{13}C$-labeled species in $^{13}C$ (1D) INADEQUATE NMR experiments (Fig. 8B). No evidence of major $^{13}C$-$^{13}C$-coupled species has been observed in the carbohydrate region during investigations of material obtained at different stages of cell growth. The major recipient of the double label was the protein fraction (predominantly isoleucine, leucine, and glutamate). Two peaks, however, at 67 ppm (single asterisk) and 74 ppm (double asterisks) (Fig. 8A and B), were labeled enough to be correlated with the flow of $^{13}C$ label back into the small molecule pool. These species may be phosphorylated $C_3$ intermediates attached to some macromolecule. These peaks were detectable in the pellet samples taken immediately after incubation of the cells at 50°C (Fig. 8A and B). After resumption of growth at 62°C, the peaks at 67 and 74 ppm in the $^{13}C$ spectrum of the pellet disappeared rapidly and could not be detected after 8 h in either the normal (Fig. 8C) or the 1D INADEQUATE (Fig. 8D) NMR spectrum. There was no evidence of the accumulation of these labeled species in the cell pellet during exponential growth at 62°C in the presence of [U-$^{13}C$]acetate but without prior incubation at 50°C (Fig. 9).

## DISCUSSION

The investigation of specific $^{13}C$ intensities at different stages of cell growth of phosphorylated three-carbon small molecules has shown that at 62°C, 2-PG is the synthetic precursor of 2,3-DPG and cDPG is synthesized from 2,3-DPG. Rapid interconversion of $^{13}C$ label among these species suggests that the degradation of cDPG to 2-PG via 2,3-DPG also takes place, and this reverse direction can dominate under certain conditions. The two directions are probably catalyzed in vivo by two sets of enzymes, consistent with in vitro studies of cDPG formation in *M. fervidus* (15). In that methanogen, cDPG and 2,3-DPG are produced from 2-PG in two subsequent ATP-dependent reactions catalyzed by 2-PG kinase and cDGP synthetase. Under these conditions, 2-PG kinase catalyzed 2,3-DPG synthesis unidirectionally. The reverse reaction, from cDPG to 2-PG via 2,3-DPG, was not detected either in crude extracts or with purified enzymes from *M. fervidus*. Investigations of ATP formation in cell extracts of *M. thermoautotrophicum* (23) showed that the degradation of 2,3-DPG to 2-PG and PEP to pyruvate could be used to generate ATP under hydrogen-limited conditions. Under these conditions, cell carbon synthesis via acetyl coenzyme A would not be feasible, and the conversion from cDPG to pyruvate could occur. The

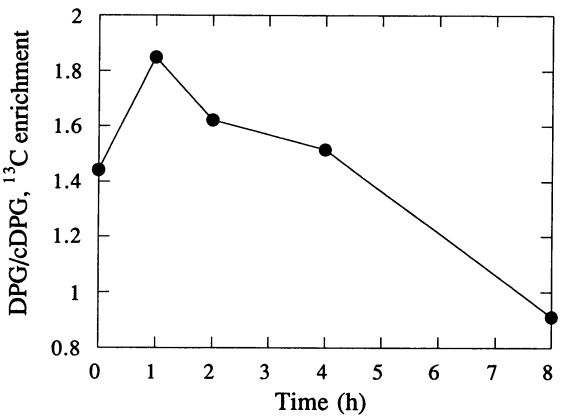


FIG. 10. Specific $^{13}C$ intensity of 2,3-DPG normalized to the specific $^{13}C$ intensity of cDPG in ethanol extracts of exponentially growing cells of *M. thermoautotrophicum* as a function of time after the start of the [U-$^{12}C$]acetate chase.

enzyme responsible for the unidirectional degradation of cDPG to 2,3-DPG, 33-kDa cDPGase, was purified from *M. thermoautotrophicum* (19) and is a protein distinct from cDPG synthetase.

The present studies provide evidence that all these reactions observed in vitro occur in vivo in *M. thermoautotrophicum* at 62°C. However, formation and degradation through 2-PG are not the only means for the synthesis and consumption of cDPG. The relative distribution of $^{13}C$ label between 2,3-DPG and cDPG is not consistent with postulated dead-end synthetic schemes (19, 23) in which cDPG is just a storage pool either for gluconeogenesis (19) or for ATP formation (23). In the presence of continuous $^{13}C$ label flow from 2,3-PG to cDPG, 2,3-DPG becomes 40% more enriched than cDPG. Instead of a decrease in that ratio in the first stage of the [$^{12}C$]acetate chase, the cDPG/2,3-DPG specific $^{13}C$ content ratio actually increases after 1 h and only then gradually decreases (Fig. 10). The most plausible explana-

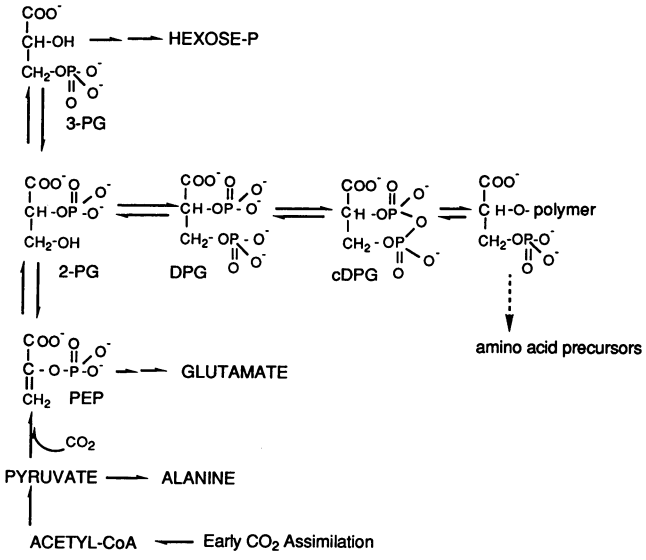


FIG. 11. cDPG as an intermediate in a metabolic branch of the gluconeogenesis pathway in *M. thermoautotrophicum*. ACETYL-CoA, acetyl coenzyme A.

tion is that cDPG is the component of a new synthetic pathway that branches from gluconeogenesis by converting 2-PG to cDPG via 2,3-DPG and that this pathway finally is used for macromolecular synthesis and degradation (Fig. 11). The $^{13}C$-labeled polymeric material that correlates with cDPG and 2,3-DPG $^{13}C$ label flow has chemical shifts similar to those of 3-PG (68 and 73 ppm). This result, coupled with the fact that there is no significant change in the C-2–C-3 labeling pattern of observed molecules during the different stages of cell growth, tentatively suggests that the polymeric species could be 3-PG linked via the C-2 atom. It is possible that this biosynthetic pathway connects the phosphorylated three-carbon intermediate pool of gluconeogenesis with some precursors of amino acid synthesis. In that case, when methanogenesis can supply acetyl coenzyme A in abundance, a large intracellular cDPG pool would help to balance carbohydrate and protein syntheses. On the contrary, under starvation conditions, it would facilitate the balance between macromolecular degradation and ATP synthesis.

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## REFERENCES

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:**260–296.
2. **Bax, A., R. Freeman, and T. A. Frenkiel.** 1981. An NMR technique for tracing out the carbon skeleton of an organic molecule. J. Am. Chem. Soc. **103:**2102–2104.
3. **Daniels, L., R. Sparling, and C. D. Sprott.** 1984. The bioenergetics of methanogenesis. Biochim. Biophys. Acta **768:**113–163.
4. **Evans, J. N. S., D. P. Raleigh, C. J. Tolman, and M. F. Roberts.** 1986. $^{13}C$ NMR spectroscopy of *Methanobacterium thermoautotrophicum*. Carbon fluxes and primary metabolic pathways. J. Biol. Chem. **261:**16323–16331.
5. **Evans, J. N. S., C. J. Tolman, S. Kanodia, and M. F. Roberts.** 1985. 2,3-Cyclopyrophosphate in methanogens: evidence by $^{13}C$ NMR spectroscopy for a role in carbohydrate metabolism. Biochemistry **24:**5693–5698.
6. **Evans, J. N. S., C. J. Tolman, and M. F. Roberts.** 1986. Indirect observation by $^{13}C$ NMR spectroscopy of a novel $CO_2$ fixation pathway in methanogens. Science **231:**488–491.
7. **Eyzaguirre, J., K. Jansen, and G. Fuchs.** 1982. Phosphoenolpyruvate synthetase in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **132:**67–74.
8. **Fuchs, G., H. Winter, and E. Stupperich.** 1983. Enzymes of gluconeogenesis in the autotroph *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **136:**160–162.
9. **Hensel, R., and H. Konig.** 1988. Thermoadaptation of methanogenic bacteria by intracellular ion concentration. FEMS Microbiol. Lett. **49:**75–79.
10. **Jones, W. J., D. P. Nagle, and W. B. Whitman.** 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51:** 135–177.
11. **Kanodia, S., and M. F. Roberts.** 1983. Methanophosphagen: unique cyclic pyrophosphate isolated from *Methanobacterium thermoautotrophicum*. Proc. Natl. Acad. Sci. USA **80:**5217–5221.
12. **Keltjens, J. T., L. Daniels, H. G. Jannsen, P. J. Borm, and G. D. Vogels.** 1983. A novel one-carbon carrier (carboxy-5,6,7,8-tetrahydromethanopterin) isolated from *Methanobacterium thermoautotrophicum* and derived from methanopterin. Eur. J. Biochem. **130:**545–552.
13. **Keltjens, J. T., and C. van der Drift.** 1986. Electron-transfer reactions in methanogens. FEMS Microbiol. Lett. **39:**259–301.
14. **Konig, H., E. Nusser, and K. O. Stetter.** 1985. Glycogen in *Methanolobus* and *Methanococcus*. FEMS Microbiol. Lett. **28:**265–269.
15. **Lehmacher, A., A. B. Vogt, and R. Hensel.** 1990. Biosynthesis of cyclic 2,3-diphosphoglycerate. Isolation and characterization of 2-phosphoglycerate kinase and cyclic 2,3-diphosphoglycerate synthetase from *Methanothermus fervidus*. FEBS Lett. **272:**94–98.
16. **Leigh, J. A., K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Structure of methanofuran, the carbon dioxide reduction factor of *Methanobacterium thermoautotrophicum*. J. Am. Chem. Soc. **106:**3636–3640.
17. **McBride, B. C., and R. S. Wolfe.** 1971. A new coenzyme of methyl transfer, coenzyme M. Biochemistry **10:**2317–2324.
18. **Pellerin, P., B. Gruson, G. Prensier, G. Albagnac, and P. Debeire.** 1987. Glycogen in *Methanothrix*. Arch. Microbiol. **146:**377–381.
19. **Sastry, M. V. K., D. E. Robertson, J. A. Moynihan, and M. F. Roberts.** 1992. Enzymatic degradation of cyclic 2,3-diphosphoglycerate in *Methanobacterium thermoautotrophicum*. Biochemistry **31:**2926–2935.
20. **Seeley, R. J., and D. E. Farney.** 1983. A novel diphospho-P,P′-diester from *Methanobacterium thermoautotrophicum*. J. Biol. Chem. **258:**10835–10838.
21. **Tolman, C. J., S. Kanodia, L. Daniels, and M. F. Roberts.** 1986. $^{31}P$ NMR spectra of methanogens: 2,3-cyclophosphoglycerate is detectable only in methanobacterium strains. Biochim. Biophys. Acta **886:**345–352.
22. **Turner, J. D., and G. Rouser.** 1970. Precise quantitative determination of human blood lipids by thin-layer chromatography. 1. Erythrocyte lipids. Anal. Biochem. **38:**423–436.
23. **Van Alebeck, G.-J. W. M., C. Klaassen, J. T. Keltjens, C. van der Drift, and G. T. Vogels.** 1991. ATP synthesis from 2,3-DPG by a cell-free extract of *M. thermoautotrophicum* (strain ΔH). Arch. Microbiol. **156:**491–496.
24. **Whitman, W. B.** 1985. The bacteria, vol. 8, p, 4–65.