DOE/ER/13995--T3

# Progress Report
# The Role of Purine Degradation in Methane Biosynthesis and Energy Production in *Methanococcus vannielii*

## ACCOMPLISHMENTS

1) Characterization of a purine degrading pathway in *Methanococcus vannielii*. The pathway is similar to that described for *Clostridia*, and is shown in Figure 1 at the end of the report. The *M. vannielii* pathway differs in a few respects from the *Clostridial* pathway. The pathway of *Clostridia* uses tetrahydrofolic acid (THF), whereas the pathway of *M. vannielii* uses tetrahydromethanopterin ($H_4$MPt) as a cofactor in the transfer of both the formimino moiety of formiminoglycine and apparently in the cleavage of glycine by a glycine decarboxylase type mechanism that is dependent upon at least $H_4$MPt and either $NAD^+$ or $NADP^+$ (both seem to work in crude extracts). Known glycine decarboxylases catalyze the following reaction:

$$\text{glycine} + \text{THF} + \text{NAD(P)}^+ \rightarrow CO_2 + NH^3 + N^5,\ N^{10}\text{-methyleneTHF} + \text{NAD(P)H} + H^+$$

We have developed an assay for this reaction in the *M. vannielii* system based on the release of $CO_2$ from L-[1-$^{14}$C]glycine. We plan to purify this enzyme complex so that we may better characterize the involvement of $H_4$MPt. We also have developed assays for the first two enzymes of this pathway with an eye toward their purification and characterization. If this pathway is regulated, we would expect the regulation to occur at this step, hence our interest in this enzyme. The assay for xanthine amidohydrolase (Figure 1, E3) employs C-18 reverse phase separation of xanthine from the product of the reaction, 4-ureido-5-imidazolecarboxylic acid. This product is readily identifiable by its characteristic pH-dependent UV spectrum. The assay for the reaction catalyzed by the next enzyme (Figure 3, E4) is based on the release of $^{14}CO_2$ from [2-$^{14}$C]4-ureido-5-imidazolecarboxylic acid prepared enzymatically from [2-$^{14}$C]xanthine. We wish to study this enzyme because of its interesting enzymology. We have determined that the enzyme from *M. vannielii* requires $Mn^{+2}$ or $Fe^{+2}$ for activity. The site of cleavage, the ureido moiety and the requirement for a divalent transition metal are reminescent of urease, which has $Ni^{+2}$ as a component of its active site. We have waited until we have developed assays for all three of these enzymes in order that we may purify them concurrently. One of our bottlenecks is production of cellular material, which is also the only source of methanogen coenzymes. This plan will save our valuble supply of cells.

2) Relationship of purine degradation to methanogenesis. The degradation of xanthine by *M. vannielii* produces five $C_1$ units. The C-2, C-4 (via glycine), and C-6 atoms of xanthine are liberated as $CO_2$, and the C-5 (via glycine) and C-8 atoms are transferred to $H_4$MPt. Since our initial studies have been performed with crude extracts, we cannot be absolutely sure what state of $H_4$MPt is

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formed on transfer of the carbon atoms (due to interconversions of the various forms of the cofactor catalyzed by enzymes in the crude extract), but based on analogous reactions it is likely that C-5 of xanthine (C-2 of glycine) and $H_4$MPt generate $N^5$, $N^{10}$-methylene-$H_4$MPt, and that C-8 of xanthine and $H_4$MPt generate $N^5$, $N^{10}$-methenyl-$H_4$MPt. Since both of these cofactor forms are also components of the methane biosynthetic pathway or are required in other biosynthetic steps (such as TMP synthesis from dUMP), it was reasonable to assume that all of these carbon atoms may enter the methane biosynthetic pathway or may be used to synthesize cellular material. We have found that the former case is true by direct evidence, and we know that the latter is true because $^{14}C$ from in various positions in xanthine and glycine are incorporated into acid insoluble material with time. We have yet to assess the extent to which purines can contribute carbon for growth and methanogenesis. The main limitation to this appears to be their limited solubility, especially at neutral pH. We did not appreciate this initially because *M. vannielii* grows very well with formate as a carbon source in the pH range of 8.0 to 8.8. Also there is, not surprisingly, a temperature effect on the solubility of these compounds, however this temperature effect is not as pronounced in going from, for example 25 to 40°C, as the substantial increase in solubility of xanthine, guanine, or hypoxanthine seen when going from pH 7.0 to 8.5. We considered that there might be a growth retriction due to purine insolubility when we had problems trying to grow *M. vannielii* at 30°C with uric acid as sole nitrogen source and $CO_2/H_2$ as carbon and energy source at pH 7.0. This growth restriction is due to the fact that most purines and their respective nucleosides have pK's at slightly basic pH (xanthine and xanthosine, 7.4; guanine and guanosine, 9.0; hypoxanthine and inosine, 8.9). This means that *M. vannielii* growing with formate as carbon source is better suited for utilizing purines and nucleosides than is a microorganism which grows best near pH 7.0. Increasing the pH to 8.5 does not substantially affect the solubility of uric acid (pK, 11.3), however. For optimal growth of microorganisms in the laboratory with uric acid, a chemical trick must be applied.

Solubilizing uric acid has evidently been the source of several of our problems. We have gotten inconsistent results when we have tried to grow *M. vannielii* with uric acid as sole nitrogen source. Sometimes we would get no growth, other times *M. vannielii* would grow fine. Different batches of cells would have varying levels of xanthine dehydrogenase, the enzyme necessary for uric acid utilization, that is induced by the presence of some as yet undetermined level of uric acid. We have resolved the problems of uric acid insolubility. A look at past literature dealing with growth of other anaerobes on uric acid indicates that others must have occasionally had solubility problems, but they usually solved the problem empirically, and were aided by the more rapid growth of their bacteria. We found that the trick to growing bacteria on uric acid is to insure that the medium is supersaturated with uric acid when inoculated. When the growth medium is put together, uric acid (2 to 5 mM) is added first, the medium is heated, and NaOH is added until the purine dissolves. Other medium components are added, the pH is lowered to approximately 8.0 to 8.5, and the

medium is cooled to around 40°C. At this point, uric acid is supersaturating. If inoculation with the microorganism is done at this point, uric acid can evidently be metabolized so that it may be used as sole nitrogen source for growth. During the next few days uric acid solubility goes toward the equilibrium condition and the compound falls out of solution. However, this has usually been a long enough time to allow *M. vannielii* to use the compound as a source of nitrogen. Since tables of purine solubilities that were available to us are not complete with respect to all temperatures and pH values of interest, we carried out solubility tests of various purines at different temperatures and pH values in our standard *M. vannielii* growth medium to confirm our suspicions that the pH and temperature dependent solubility problems of growth on purines might account for the variability in growth we had been seeing. Our results supported the conclusions made in the above discussion. Additionally our results may explain why methanogens that grow at mesophilic temperatures near pH 7, and that can utilize purines as nutrients at lower concentrations (approximately 0.1 M), might not ever encounter purines at levels above 1 mM where *M. vannielii* is able to used them as a general source of nitrogen.

Purine and purine nucleoside utilization by *M. vannielii*. We have found that *M. vannielii* does either of two things with purines it takes from its growth medium. It either degrades them according to the pathway described later or it converts them to nucleotide monophosphates which are used for a variety of cellular needs. The incorporation of various purines into the purine nucleotide pool has also been noted for other methanogens. We performed experiments wherein *M. vannielii* was grown in the presence of 20 mM $NH_4^+$ and 50 µM of either [5′-$^3$H]guanosine, [U-$^{14}$C] guanosine, or [8-$^{14}$C]guanine. The specific activities of GMP, AMP, CMP, and UMP isolated from RNA, and Factor 420 ($F_{420}$) and tetrahydromethanopterin ($H_4$MPt) were then analyzed and compared to the specific activities of the added guanine or guanosine. Additionally, the amount of labeled compound taken into the cells was determined. 16%, 38%, and 60% of the labeled atoms that originated in [5′-$^3$H]guanosine, [U-$^{14}$C]guanosine, and [8-$^{14}$C]guanine, respectively, were found inside the cells. These values could be underestimates of the amounts taken up if a significant amount of the fragile cells lysed during growth and harvest. The results from specific activity measurements are summarized in Table 1.

Several conclusions can be made from these results. 1) At 50 µM guanosine is not take up as completely as guanine (see above). That less of the $^3$H-label than the $^{14}$C-label from guanosine was found in the cells could be due to ex-perimental variation or due to the fact that $^3$H is more readily exchangeable, so it could be lost to the medium as $CH_4$ or $H_2O$. 2) When guanosine is taken into the cell, the ribose is readily cleaved from guanine. When the label is only in ribose (5′-$^3$H-label), one sees that the specific activity of GMP is 1/12 of that when the label is in both the ribose and guanine (U-$^{14}$C-label). This difference is not accounted for by the uptake/exchange difference in these two compounds, which was 16% vs. 38%, a ratio of 1/2.4. 3) Although some label is found in all nucleotides in each of these experiments, there is no significant conversion of guanine, guanosine, or GMP to AMP. This

is particularly evident in the [8-$^{14}$C] guanine study. 4) The added 50 µM guanine accounts for 71% of the GMP isolated from RNA. This indicates that *M. vannielii* takes up guanine avidly, and that de novo guanine nucleotide synthesis has been significantly inhibited or repressed by the guanine that was transported. 5) Synthesis of $H_4$MPt and $F_{420}$ from guanine nucleotides with loss of the 8-carbon (at least in the case of $H_4$MPt) is affirmed. 6) Ammonium ion at a concentration of .20 mM does not inhibit guanine (10 to 50 µM) uptake. 7) No conclusive statement can be made about the amount of guanine degradation that occurred in these experiments, because $CO_2$ and $CH_4$, which should be the main depositories for label, were not analyzed. 8) We cannot explain the relatively high specific activity of the $F_{420}$ from the [8-$^{14}$C]guanine experiments, however this value is close to the 3% value obtained by previous investigators (Jaenchan, et al. 1984. Arch. Microbiol. 137:362-365).

Table 1

| | [5'-$^3$H]G-R ($5.20 \times 10^{12}$) | [U-$^{14}$C]G-R ($1.84 \times 10^{12}$) | [8-$^{14}$C]G ($2.78 \times 10^{12}$) |
|---|---|---|---|
| CMP | $4.9 \times 10^9$ (0.10) | $4.1 \times 10^9$ (0.22) | $2.9 \times 10^8$ (0.01) |
| AMP | $5.3 \times 10^9$ (0.10) | $4.6 \times 10^9$ (0.25) | $5.7 \times 10^9$ (0.20) |
| UMP | $4.7 \times 10^9$ (0.09) | $3.1 \times 10^9$ (0.17) | $2.4 \times 10^8$ (0.01) |
| GMP | $1.0 \times 10^{10}$ (0.20) | $1.2 \times 10^{11}$ (6.7) | $2.0 \times 10^{12}$ (71) |
| $F_{420}$ | $2.3 \times 10^{10}$ (0.44) | $2.0 \times 10^{11}$ (11) | $1.3 \times 10^{11}$ (4.6) |
| $H_4$MPt | $1.2 \times 10^{10}$ (0.24) | $2.1 \times 10^{11}$ (12) | $8.1 \times 10^9$ (0.29) |

[A]The values at the top of the table are the compounds (with their sp. act. below) added to cultures of *M. vannielii*. The values in the table are the sp. act. of the various compounds listed at the left, which were purified from the cells harvested from the cultures and analyzed. The number in parentheses next to the sp. act. is the percent of the sp. act. of the compound at left compared to the sp. act. of the added compound.

Xanthine Dehydrogenase. This enzyme is responsible for the interconversions, through anaerobic oxidations or reductions, of certain purines. We have known for several years that the enzyme from *M. vannielii* can utilize either uric acid, xanthine, or hypoxanthine as a substrate. We purified this enzyme some time ago, but had only a partially characterized it due to a lack of material. Purification of additional quantities in the interim has been thwarted by a lack of suitable (with high enough specific activity) cellular material, the absence of the previously used

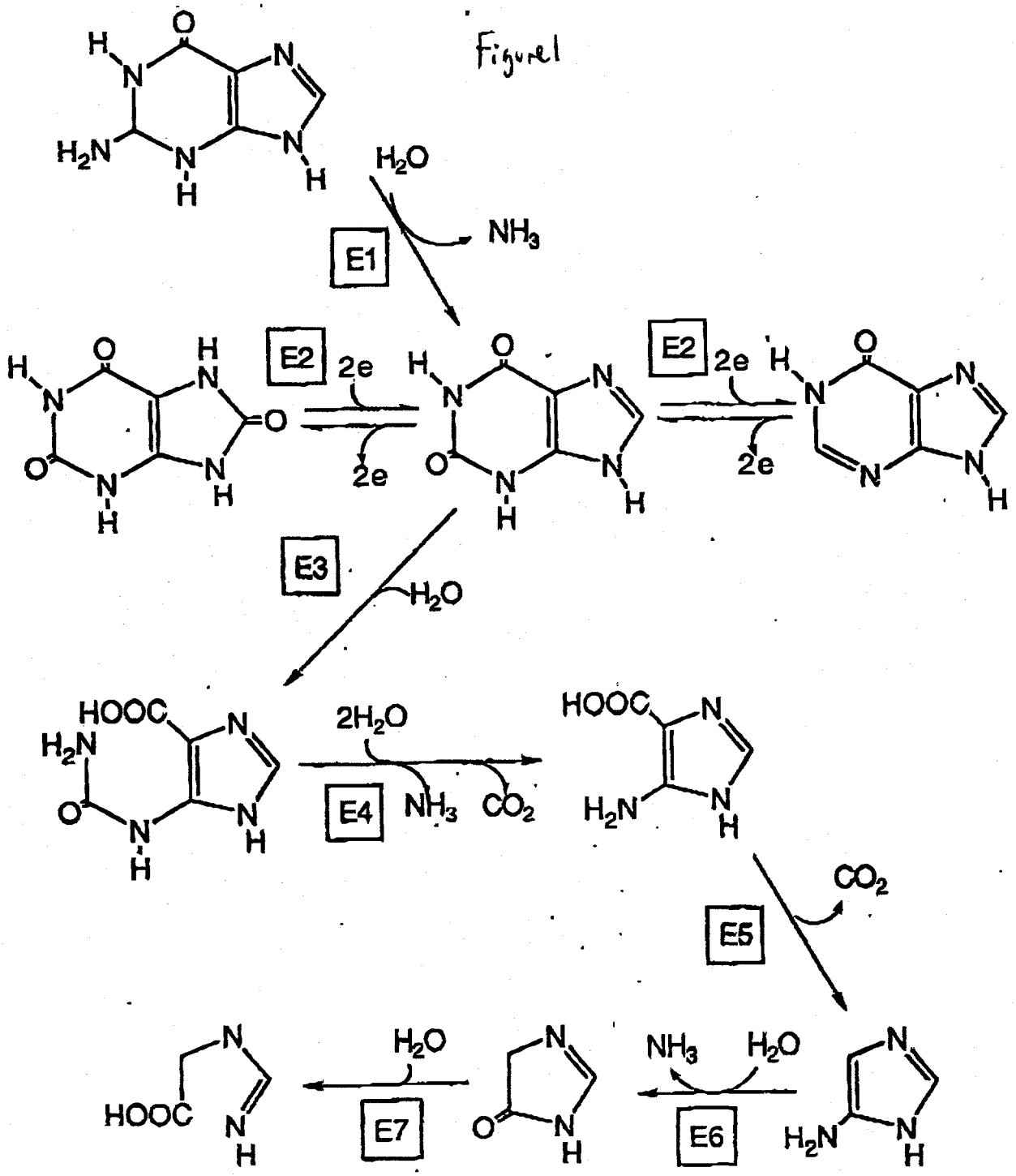
HPLC system, and the desire to perform certain studies which require a relatively large quantity of enzyme, for example the characterization of the form of molybdenum cofactor present in the enzyme. Our characterizations of the enzyme have shown that it has three kinds of subunits of approximate molecular weights 91,200, 34,700, and 15,900. Based on native polyacrylamide gels, which indicate a molecular weight of approximately 165,000, the subunit composition is estimated to be either 1:2:1, 1:1:2, or 1:1:3. Efforts are ongoing to obtain a better value for the native molecular weight, so that we may know the exact subunit composition. We have not yet completed the metal analysis of the enzyme, because we are currently performing kinetic studies and do not yet have enough enzyme to incinerate in a metal determination. So that we may conserve enzyme, xanthine dehydrogenase from these studies is being saved for the various analyses which require destruction of the enzyme. Uric acid induces synthesis of this xanthine dehydrogenase (XDH). In fact the enzyme is undetectable in cells that have not been exposed to uric acid. XDH catalyzes the reversible oxidation and reductions of uric acid to xanthine to hypoxanthine. The kinetic experiments we have done so far tend to indicate that the oxidation of xanthine involves a disproportionation to uric acid and hypoxanthine when there is no other electron acceptor present. In order to complete these studies we will evidently need to HPLC purify out purines prior to use, since those commercially available contain various amounts of other purines.

Nitrogen sources of *M. vannielii*. We decided to see if other biochemicals other than purines and ammonia can supply the nitrogen requirement for *M. vannielii*. We have found that a mix of 17 amino acids (the usual 20 minus glutamine, asparagine, and cysteine) added at 50 mg/L each, can supply enough nitrogen to *M. vannielii* grown in our standard 1.5% formate medium to replace $NH_4^+$ and allow a reduced, but significant level of growth as measured by the increase in protein in the culture. In the course of these studies, we also discovered that even at high growth rates, *M. vannielii* uncouples methane synthesis from growth. When amino acids replaced ammonium ion as the nitrogen source, although the organism grew only about 1/3 as much as cultures with ammonium ion (10 mM) and only about 1/4 as much as cultures with ammonium ion and amino acids, the methane production measured in each culture was approximately the same (within an average of ±5%). Also a culture with no nitrogen added (so that the only nitrogen source was the carryover in the 10% inoculum) grew very little, only 1/10, 1/30, and 1/40 as much as the amino acids only culture, the ammonium ion only culture, and the ammonium ion plus amino acids culture, respectively. Despite this slight amount of growth, the culture still produced 1/5 the amount of methane as seen in the other three cultures. The no nitrogen culture had a ratio of methane produced to growth seven times higher than the culture with ammonium ion and amino acids. Further investigation found that each of the 17 amino acids alone, when acting as the sole nitrogen source at 20 mM, allowed for growth and methanogenesis. However, in these cases the ratio of methanogenesis to growth was even higher than in the 17 amino acid mix. Apparently all amino acids may deaminated to enough of an extent to allow for some growth. These results com-

plete our picture of how *M. vannielii* adjusts its intermediary metabolism to accomodate its nitrogen requirement.

Reactions which require $H_4MPt$ in *M. vannielii*. From evidence we have gathered in our studies of purine degradation in *M. vannielii* it is likely that intermediary metabolism of $C_1$ units in and out of the main methane biosynthetic pathway in this organism is mediated by $H_4MPt$. In Figure 2 some of these reactions are shown branching off of the main pathway in much the same way that various pathways flow in and out of, for example, the Emden-Meyerhof-Parnas pathway of glucose utilization. We have shown that reactions 3 and 5 take exist. Others have shown the existence of reaction 6. For a time we were interested to see if reactions 7 and 8 could be carried out in *M. vannielii*. If so, this would have been the first demonstration of substrate level phosphorylation in a methanogen. It would also have been the only demonstration of $N^{10}$-*formyl*-$H_4MPt$. The analogous THF compound is well known and is used in these two reactions as well as in formylation of methionine in eubacterial protein synthesis. We have been unable to demonstrate any ATP dependent formylation of $H_4MPt$, and archaebacteria do not formylate methionine. Is there a role for the $N^{10}$-form of this cofactor? There is only one more known role, and we are presently checking it out. This role is in purine biosynthsis. That methanogens do not carry out the substrate level phosphorylation reaction is further evidence that more than one ATP might be derived from each methane molecule formed. This is thought to be the case due to the necessity of recovering more than the one ATP expended to activate acetate in the case of methanogens grown with acetate as carbon source. Perhaps methanogens do not utilize the substrate level phosphorylation step since the do better energetically simply by sending the $C_1$ unit into methane synthesis.

Figure1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E2
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2e
E2
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2e
E3
H2O
HOOC
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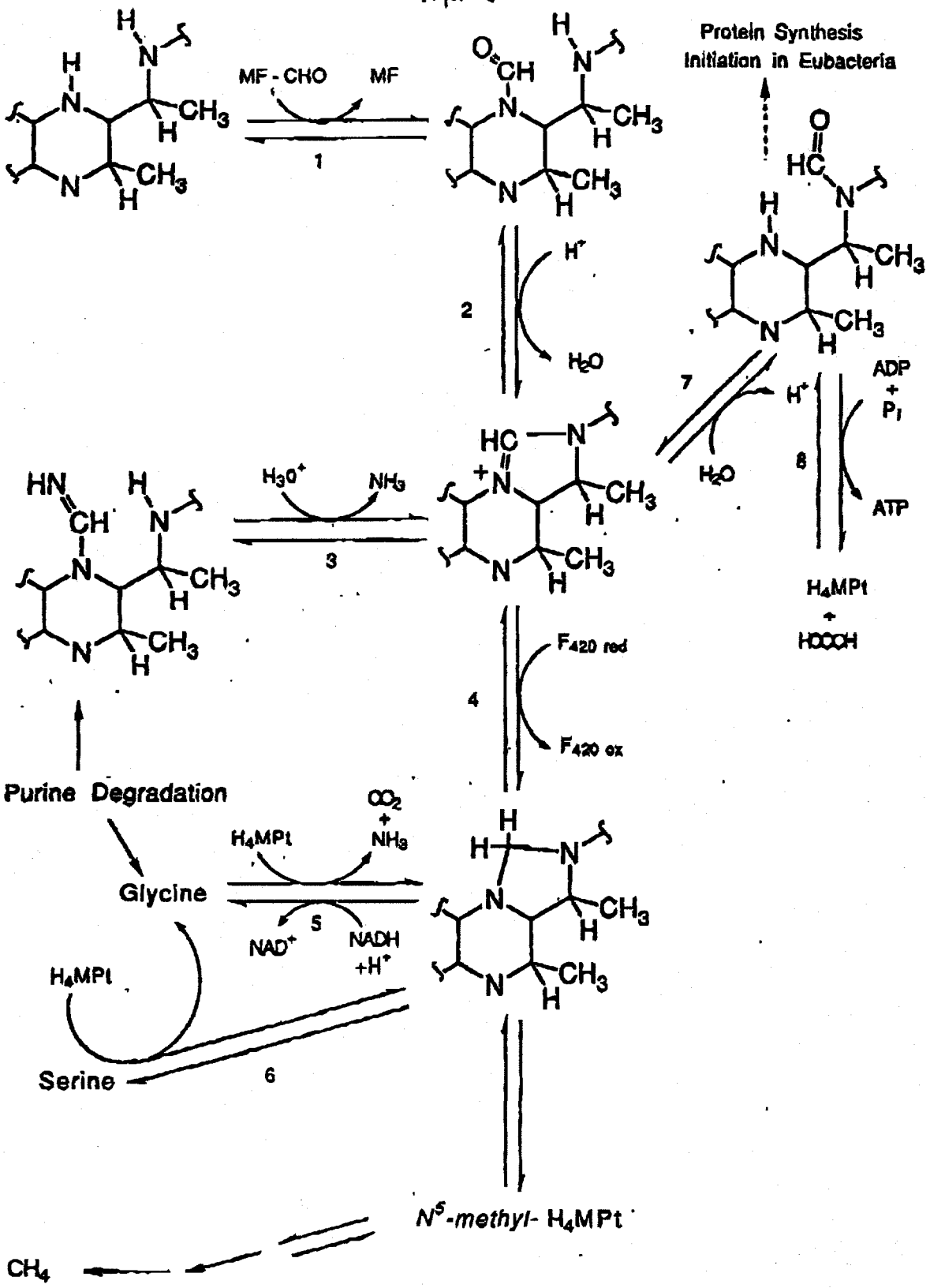
E5
H2O
E7
NH3
H2O
E6

figure2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2
H+
H2O
Protein Synthesis
Initiation in Eubacteria
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ADP
+
Pi
8
ATP
H4MPt
+
HCOOH
H3O+
NH3
3
F420 red
4
F420 ox
Purine Degradation
Glycine
CO2
+
NH3
H4MPt
NAD+
5
NADH
+H+
H4MPt
Serine
6
N5-methyl- H4MPt
CH4