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Biochemistry of methanogenesis

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The recent and unexpected finding that methanogenic bacteria occupy an isolated biochemical island in the sea of procaryotes has added a touch of excitement to the study of these organisms[1]. This island is defined by such diverse biochemical qualities as: a very restricted range of oxidizable substrates coupled to the biosynthesis of methane; synthesis of an unusual range of cell-wall components; synthesis of biphytanyl glycerol ethers as well as high amounts of squalene; synthesis of unusual coenzymes and growth factors; synthesis of rRNA that is distantly related to that of typical bacteria; possession of a genome size (DNA) approaching ⅓ that of *E. coli.*

Our purpose here is to focus on those aspects of the biochemistry of methanogens that are related directly to the biosynthesis of methane. Barker[2] and his students made fundamental contributions to knowledge of the mechanism of methane formation. They showed that for certain methanogenic bacteria carbon dioxide is the precursor of methane. That is, carbon dioxide serves as the final electron acceptor and is reduced to methane. A stepwise scheme was postulated for this process in which 8 electrons were consumed in the reduction of 1 molecule of carbon dioxide. In another contribution they documented that in certain other methanogenic bacteria the

methyl group of acetate or methanol was converted to methane. By use of deuterated acetate or methanol they proved that the hydrogen or deuterium atoms on the methyl carbon remained attached to the carbon atom. So the methyl group was transferred intact and was reduced, accepting 1 proton from the medium.

These 2 mechanisms represent the 2 major routes for methane formation in nature. In sediment and sludge digesters 60–70% of methane is formed from the methyl group of acetate, whereas 30–40% of the methane arises from reduction of carbon dioxide. These 2 routes of methane formation also reflect the 2 major routes of substrate oxidation by methanogenic bacteria. Oxidation of hydrogen or formate is coupled to the reduction of carbon dioxide. All of the 13 species of methanogens now in pure culture are able to oxidize hydrogen and reduce carbon dioxide to methane[1]. The acetophilic methanogens are represented in pure culture at present only by *Methanosarcina,* an organism that is the most metabolically diverse methanogen, converting hydrogen and carbon dioxide as well as methanol, acetate, and methylamines to methane. Other acetophilic methanogens are known, and some of them are in a highly purified stage of culture[3].

Of the 2 major substrate systems of methanogens, a) the hydrogen-carbon dioxide (and formate) system and, b) the acetate system, the former has yielded to fractionation and biochemical studies of subcellular components, whereas the latter is poorly studied. The reason for this is very simple; hydrogenophilic methanogens have yielded to mass culture; acetophilic methanogens are difficult to mass cultivate, having a long generation time and poor cell yield. Of the minor substrate systems methanol produces a better growth response for *Methanosarcina* than does acetate; growth on methylamines is a relatively recent finding[4].

When we began to study the reduction of carbon dioxide to methane by use of $^{14}CO_2$, counts were found in the reaction mixture associated with a small, acidic molecule. McBride fractionated this compound and showed that it was converted to methane. He named this compound coenzyme M, since it was involved in methyl transfer[5]. Its structure was determined by Taylor[6] to be 2-mercaptoethanesulfonic acid. This molecule accepts a methyl group to become 2-(methylthio)ethanesulfonic acid which is the substrate for the methylreductase of methanogens. This molecule is unique in that it is the smallest of the coenzymes, having the most oxidized sulfur atom on one end and the most reduced sulfur atom on the other end separated by a CH_2CH_2 moiety. *Methanobrevibacter ruminantium*[1] (formerly *Methanobacterium ruminantium,* Ml) requires coenzyme M as a growth factor[7], a vitamin. So this compound has a classical vitamin-coenzyme relationship. To test the specificity of this compound Romesser and Gunsalus[8] synthesized a wide variety of analogues and derivatives that were tested in cell extracts as well as in the *M. ruminantium* vitamin assay system.[9]. If substitutions were made for either sulfur atom, the derivatives were neither active as vitamin nor coenzyme. For example, taurine or isethionic acid were completely inactive. Bromoethanesulfonic acid and chloroethanesulfonic acid were powerful inhibitors of methanogenesis at 10^{-6}M. Addition of an extra C_1moiety between the sulfur atoms destroyed activity. Methyl, ethyl, or hydroxymethyl-coenzyme M could be metabolized. For example, extracts produced ethane from ethyl-coenzyme M at 20% of the rate that methane was produced from methyl-coenzyme M[8].

To study the biosynthesis of coenzyme M we decided to use *Escherichia coli,* but to our surprise the coenzyme was not to be found in this organism. This seemed odd, since water-soluble vitamins and coenzymes were known to have a universal distribution in the biological world. Balch[9] then performed a long series of careful experiments documenting the distribution of coenzyme M. A wide variety of organisms and tissues were extracted under a variety of conditions. The sensitivity of the vitamin assay was 10 pmoles. The results were clear-cut; coenzyme M was not found elsewhere but was present in all methanogens tested. We were forced to conclude that methanogens were different. This was the first indication (besides the fact that methanogens produce methane) that these organisms had unique properties.

With the discovery of methyl-coenzyme M it became possible to study the methylreductase system, and it is in this area that most progress has been made. Taylor[6] developed a small reaction vial that was sealed with a rubber septum and in which the volume of the reaction mixture was 0.25 ml. Components were added by syringe, and the gas atmosphere was made anaerobic by use of gassing needles. As methane was formed it escaped into the atmosphere of the reaction vial from which samples could be transferred to a gas chromatograph by syringe. For the methyl group of methyl-coenzyme M to be converted to methane, hydrogen and ATP were required.

To fractionate oxygen-sensitive components of methanogens we found it necessary to take exceptional care to exclude oxygen. Gunsalus developed a system that employed an anaerobic Freter-type chamber[10] that contained an atmosphere of 97% nitrogen and 3% hydrogen. In this flexible plastic chamber oxygen that diffused through plastic walls was scrubbed out by circulating the gas atmosphere over palladium catalyst. Deoxygenated solutions were transferred into the chamber through the air lock. A chromatographic column with o-ring seals was poured aerobically. The inlet and outlet of the column were connected by thick polyethylene tubing to connector ports in the

wall of the chamber. After pumping 3–4 column-volumes of anaerobic buffer through the column, the sample was pumped onto the column. Elution buffer was pumped through the column, with the eluate being returned to a fraction collector inside the chamber. By use of this procedure Gunsalus[11] separated the methylcoenzyme M methylreductase into 3 components. They were labeled A, B and C in order of their elution by a salt gradient from a DEAE-cellulose column. Component A was a large protein complex of about 500,000 daltons that possessed hydrogenase activity; component C was a protein of about 130,000 daltons; component B was a heat-stable cofactor. In a reconstituted system all three components were required for methane formation from methyl-coenzyme M. Components A and B were found to be oxygen-labile. At present, purification of component B is in progress but is extremely difficult; once the cofactor is exposed to oxygen it is inactivated, and no reducing conditions that we have tried regenerate any activity. The factor has no visible or UV-absorption spectrum; so the methylreductase is the only assay presently available. One of the most interesting findings in our study of the methyl-coenzyme M methylreductase was made by Gunsalus[12]. When methylcoenzyme M was added as substrate to cell extract in the presence of excess hydrogen and ATP, methane was formed in stoichiometric amounts, 1 mole of methane being formed from 1 mole of methyl-coenzyme M added. Sequential addition of more substrate yielded the same result. However, if the same experiment was carried out in the presence of hydrogen and carbon dioxide, the rate of methane formation increased 30-fold with a 12-fold increase in the amount of methane formed. At each new addition of substrate the same effect was seen again and again. This effect we have named the RPG effect after R.P. Gunsalus who discovered it; each mole of methylcoenzyme M generated an active complex through which 11 moles of carbon dioxide was activated and reduced to methane. So in some manner the terminal reaction in methane formation is coupled with the first, the activation of carbon dioxide, suggesting a definite cycle. Results of additional studies showed that the role of ATP in the methylreductase reaction was that of an activator, about 15 moles of methane being formed per mole of ATP added.

To explore the possibility that coenzyme M might be a carrier of C_1 moieties more oxidized than the methyl level Romesser[13] synthesized formylcoenzyme M, but it was not converted to methane by cell extracts. Hydroxymethylcoenzyme M also was synthesized and the C_1 moiety was converted to methane. However, hydroxymethylcoenzyme M was found to hydrolyze to formaldehyde and coenzyme M. Formaldehyde was converted to methane by cell extracts, but this conversion required coenzyme M. So the picture is a bit fuzzy; hydroxymethyl-coenzyme could be an intermediate, perhaps in a hydrophobic area of an enzyme, but definitive experiments have not yet been done.

In studying the RPG effect Romesser was able to resolve cell extracts for a factor that was required for carbon dioxide reduction to methane. Resolved extracts were not able to exhibit the RPG effect; 1 mole of methane was formed from 1 mole of methylcoenzyme M. When the factor (CDR factor) was added back, carbon dioxide was reduced to methane. This unknown factor is under study at present. The C_1 carrier at the formyl level of reduction remains unkown at the present time.

Hydrogenase was found to be a component of the methylreductase system, and no soluble electron acceptor has been implicated in this reaction. Although no direct evidence has been obtained for specific electron donors for the reduction of carbon dioxide to the formyl, formaldehyde, and methyl levels, another interesting factor, coenzyme F_{420} may be involved[14]. Coenzyme F_{420} has a strong maximal absorption at 420 nm and is a characteristic of all methanogens now in pure culture; so far it has not been found elsewhere. It is a 2-electron carrier that handles electrons at a low potential between hydrogenase and NADP, or formate and NADP. The F_{420}-NADP oxidoreductases of methanogens are rather specific for coenzyme F_{420} whereas the hydrogenases show typical activity with a wide range of natural and aritificial electron acceptors. The structure of coenzyme F_{420} was determined by Eirich to be an 8-hydroxy, 7-demethyl. 5-deaza derivative of FMN with lactyl-diglutamyl moieties attached to the phosphate of the side chain. The 5-deaza chromaphore cannot act as a semiquinone; so the coenzyme serves as a 2-electron donor.

For hydrogen-grown methanogens ATP appears to be formed by generation of a proton motive force[15]. However, ATP generation during the conversion of acetate to methane appears to be more complicated. This area has not moved in the last 20 years due to the difficulty of growing *Methanosarcina* on acetate.

To emphasize the biochemical properties of the methanogenic bacteria the following summary may be of value. These organisms are strict anaerobes that produce methane at a potential near the hydrogen electrode from mainly acetate or hydrogen and carbon dioxide; formate, methanol, and methylamines also serve as substrates for certain species. A new group of cofactors, so far found only in methanogens, includes coenzyme M[6], coenzyme F_{420}[14], undescribed factors F_{430} and F_{342}[16], component B of the methylreductase[11''], the CDR factor[13], and an unknown vitamin required for growth of *Methanomicrobium mobile* (formerly *Methanobacterium mobile).* Nature's biochemical strategy for the metabolism of small molecules has been to invent coenzymes to participate in

enzymic catalyses. When a pathway involves a novel sequence, such as the reduction of carbon dioxide to methane, then nature appears to have evolved a series of special coenzymes. The biochemical chapter on coenzymes was supposed to have been closed; we have been forced to reopen it. Methanogens appear unusual in that they apparently carry out electron transport phosphorylation in the absence of quinones, since they lack these compounds[17]. The work of Woese and colleagues has shown that the 16S rRNA of methanogens is only distantly related to typical procaryotes[18]. Kandler's laboratory has documented the wide diversity of cell-wall types among the methanogens[19]. No D-amino acids have been found, and muramic acid is absent; in one species N-acetyltalosaminuronic acid replaces muramic acid. Tornabene and Langworthy[20] have shown that the polar lipids of methanogens are non-saponifiable diphytanyl and dibiphytanyl glycerol ether-linked lipids. Squalene is found as a major component of the neutral lipids. Klotz's laboratory[21] has shown that the DNA complexity of a methanogen approaches $\frac{1}{3}$ that of *Escherichia coli*. The mechanism of carbon dioxide activation for fixation into cell carbon is unknown. It would appear that we have only scratched the biochemical surface of these interesting organisms. For example, at the present time not a single mutant or phage has been isolated. The technology for handling these organisms is now at hand, and more of nature's biochemical secrets should be revealed in the near future. Perhaps we shall eventually understand nature's strategy for maintaining such a unique group of organisms. Why have the methanogens remained as an isolated biochemical island apparently not in genetic equilibrium with the microbial world?

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Engineering, operation and economics of methane gas production

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Processing of biomass for the production of a fuel gas containing methane requires a complex system. The degree of complexity is, in part, a function of the biomass utilized. In general, this system consists of 3 main subsystems;

- Raw material preparation
- Methane fermentation
- Residue processing, utilization and/or disposal

Gas scrubbing for carbon dioxide removal to produce a gas that is essentially 100% methane is not considered in this discussion.

Certain biomass materials such as animal manure from a confined and enclosed beef feeding operation can be added directly to the fermentation subsystem without any preparation. Conversely, urban solid waste requires extensive preparation including size reduction and various separation processes for removal of those materials that have the potential for creating operational difficulties with the physical processes employed in the fermentation and residue processing subsystems.

The essence of this processing system is the methane fermentation subsystem. The ability to convert a major portion of the organic material to methane is paramount to the success of this system. This conversion efficiency has an impact on 3 separate costs. First is the raw material cost. If the biomass cost is \$20 per t, the methane cost at a 75% conversion efficiency will be about \$6.5 per 100 m^3. At a 50% conversion efficiency, the raw material cost alone is \$10 per 100 m^3.

A 2nd cost factor is associated with the reactor