Ecology of methanogenic systems in nature

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Ecological significance of methanogenesis

Decomposition in terrestrial and aquatic environments of biogenic or synthetic organic material to inorganic products (also referred to as 'mineralization') is predominantly accomplished by microbial oxidations. Under anaerobic conditions, protons, sulfur or carbon atoms are the exclusive electron sinks, products being H_2, H_2S or CH_4 respectively. In this last case, the hydrogenation of CO_2 to CH_4 or the protonation of the methyl group in acetate (or methylamins) to CH_4 are the final reactions:

$$[1]\ CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O \qquad \Delta G^{0'} = -139.1\ \text{kJ/reaction}$$

$$[2]\ CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^{0'} = -31\ \text{kJ/reaction}$$

Methanogenic bacteria are those unique microbes which can utilize anoxic ecological niches where only H_2 or such ultimate fermentation products as acetate are left over as major sources for energy gain, because other organisms have already exploited all more readily available organic compounds in the medium for ATP formation[1]. Hydrogenotrophic methanogens are also, by virtue of their specific physiological capacities, the pioneer organisms which settle those anoxic loci on earth, where only geochemical H_2 and CO_2 are available as primary substrates. - The present rate of global methanogenesis by microbial activity in terrestrial and aquatic ecosystems as well as in animal intestines has been estimated to be $5.5–11 \cdot 10^{14}$ g/year[2].

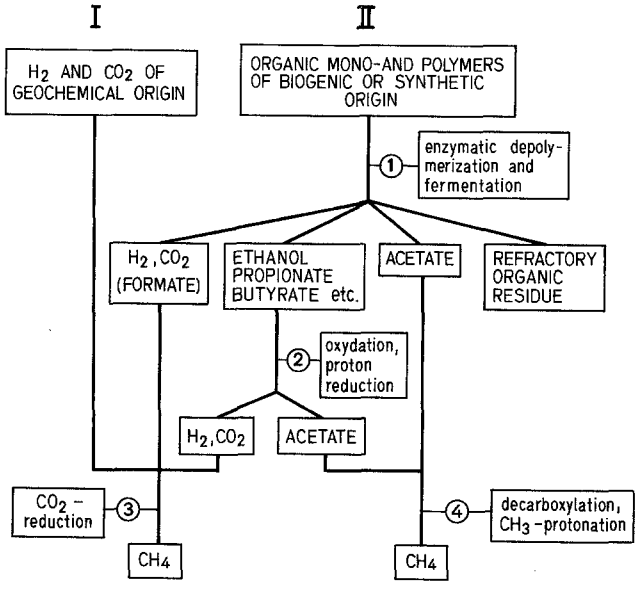
Development of the knowledge on methanogenesis in nature

The present biochemical concept for methane formation (equations [1] and [2]) is the result of two centuries of studies on the educts of methanogenesis, or more generally, on the fundamental question of the recycling of dead plants and animals under anoxic conditions. In his second letter on the origin of the 'aria infiammabile nativa delle paludi' Alessandro Volta, the discoverer of methane, wrote in November 21, 1776: '... Egli è adunque non poco verosimile che dà vegetali macerati e corrotti nell'aqua, e fors' anque dagli animali ... e non dalla pura terra o da altra fossile sostanza ...'[3]. When Béchamp in 1867[4] postulated that methane is a product of microbial fermentation, he also took it for granted that the organic substances used in his experiments (mutton meat and/or ethanol), were the immediate educts of the mixture of methane and hydrogen which he detected as fermentation products. Hoppe-Seyler[5] was the first to demonstrate that acetate decarboxylation is a mechanism of microbial CH_4-formation. This was later confirmed by Söhngen, who further suggested CO_2-reduction by hydrogen as a second reaction leading to methane[6,7]. Both authors, working with fairly crude enrichment cultures, could not exclude, however, other organic compounds as immediate substrates for methane bacteria. Barker, who first gained highly enriched (if not pure) cultures of several methane producing strains, demonstrated clearly hydrogen as an educt for microbial methane, CO_2 being the oxidant[8,9]. He further considered methane bacteria in general as the last links in anaerobic degradation chains, utilizing - besides H_2 - a very restricted and species specific number of low molecular weight end products of fermentation (e.g. formate, ethanol, acetate, propionate, butyrate)[10]. This also confirmed the earlier conclusions drawn by Buswell[11,12] from his extensive studies on sewage sludge digesters. Unequivocal confirmation of the chemolithotrophic nature of part of the methane formation in nature had still to wait, however, for the first uncontestably pure strain of such a methane producer. This was *Methanobrevibacter ruminantium,* isolated by Smith and Hungate[13] in 1958 from the rumen fluid of cattle. Their technique represented a breakthrough in anaerobic methodology and enabled other workers within a relatively short period to isolate in pure culture numerous other methane bacteria from a variety of sources. The present status of biochemical knowledge on methane formation, mostly elaborated by Wolfe within the last 12 years, is outlined in Wolfe's chapter of this review and in earlier summaries of his work (e.g. reference 14). - Interestingly enough, all these new strains were chemolithotrophic hydrogen oxidizers, with some exceptions such as *M. formicicum* which used formate, and strains of *Methanosarcina barkeri,* found to metabolize acetate or methanol besides H_2. None of the strains enriched and named by Barker which were assumed to oxidize fatty acids could be refound. Even the chief witness for organotrophic methane formation from ethanol, *M. omelianskii,* proved to be an association of a heterotrophic ethanol oxidizer (called organism S) and a strictly lithotrophic methane bacterium, *Methanobacterium bryantii*[15]. It was obvious from these findings that, on the one hand, the traditional concept of methane formation in sediments, muds, sewage

sludge digesters and similar environments from alcohols or fatty acids was no longer tenable in this generalized form. On the other hand, however, there remained unexplained such clear cut observations from material balances or experiments with isotope labelling which demonstrated methane formation from acetate up to 60% in systems with cellulose as educts, or up to 70–75% with substrates rich in fats[12,16]. There also lacked the link between the oxidation of the terminal fermentation products propionate and butyrate with methane formation in the natural ecosystems.

Hydrogenotrophic and acetotrophic methane bacteria

Hydrogen is the substrate for methane formation by the families Methanobacteriaceae, Methanococcaceae and Methanomicrobiaceae[1]. About half of the presently known species can also use formate. The metabolism of these hydrogenotrophs will be described by Wolfe in the following article. Those properties, pertinant in an ecological context, may be summarized as follows (unfortunately very few kinetic data are available): hydrogenotrophic species are found in environments with a pH around neutrality and at temperatures from about 0 °C up to 65 or 70 °C (e.g. *M. thermoautotrophicum*[14,17]). The mesophilic strain isolated in our laboratory (*M. arboriphilus* strain AZ[18,19]) absorbs hydrogen at rates up to 115 mmoles/g · h. In digested sewage sludge, containing a mixed hydrogenotrophic flora, a maximum uptake rate of ca 15 mmoles/l of sludge/h at 33 °C and at a hydrogen pressure of 0.02 atm was observed. The half rate hydrogen concentration K_s was 0.078 mmoles H_2/l[19]. These figures indicate that the actual H_2-consumption rates in such mixed methanogenic systems are about two orders of magnitude lower than the potential uptake capacity of the hydrogenotrophic community. There exists obviously a high 'buffer capacity' for maintaining hydrogen pressures at very low levels. It is shown below that this is of utmost significance ecologically.



Flow of primary substrates to CH_4 in lithotrophic (I) and organotrophic (II) methanogenic ecosystems. Educt I with reaction 3: thermal springs and lakes with intrusion of volcanic gases. Educts II (a) with reactions 1 and 3: rumen of ruminating wild or domestic animals. Educts II (b) with reactions 1 to 4: sediments, bogs, muds, sewage sludge digesters, garbage dumps, 'biogas' plants. Organism groups in reaction 1: ubiquiceous facultative and obligate anaerobic bacteria; in reaction 2: proton reducing, strict anaerobes not yet in pure culture; in reaction 3: obligate or facultative chemolithotrophic (hydrogenotrophic) methane bacteria; in reaction 4: organotrophic (acetotrophic) methane bacteria.

Acetate as a precursor of methane was already considered in the early studies of methane fermentation by Hoppe-Seyler in 1876[5] and others. Söhngen indicated two, morphologically different types of organisms as possible methane formers from acetate: 1. a coccoid, sarcina forming species and 2. a large rod, aggregating to long twisting filaments[6,7]. The sarcina type organism was enriched by Barker in 1936[8] and later gained in pure culture by several authors. The various strains are assembled in the genus *Methanosarcina* with the type species *M. barkeri*[1]. Their common property is a more or less developed capability to produce methane from acetate although lithotrophic methanogenesis from H_2 is the principle energy source for most strains. Their affinity for acetate seems to be rather small. For strain 227 for instance, a $K_{s(Ac)}$= 5 mmoles/l was reported[21] which exceeds acetate concentrations in natural environments (sediments etc.) by many times. Thus the ecological significance of the *Methanosarcina* as acetate remover is doubtful. The thermophilic strain TM1[22] might be an exception in so far as no other acetotrophic methane bacterium has been found which would eliminate acetate from high temperature environments (e.g. thermophilic sludge digesters). All experience indicates that the filamentous bacterium, already described by Söhngen and observed in mass occurrence in experimental and natural methanogenic systems by numerous authors, is mainly responsible for the fermentation of acetate to methane in psychrophilic and mesophilic environments. Barker[8] enriched such an organism and named it *Methanobacterium söhngenii;* neither he nor later authors succeeded, however, in obtaining pure cultures. It was only recently that isolation and growth in pure culture was achieved in our laboratory[23,24]. The organism agrees perfectly with the earlier descriptions by Söhngen and Barker. The bacterium transfers 98–99% of the methyl group of acetate absorbed to methane. Hydrogen is not consumed, the temperature optimum is around 37–40 °C and a pH in the range of pH 7.4–7.8 is required. In contrast to the *Methanosarcina* it has a high affinity to acetate ($K_{s(Ac)}$=0.5–0.7 mmoles/l) and a remarkable consumption rate of 1.6–2 mmoles/g biomass · h. Methane is formed from the

methyl group of acetate according to the stoichiometry of equation [2]. The organism grows very slowly (generation time at 37 °C about 8–10 days!). No other methane bacterium, consuming acetate as exclusive substrate has been reported and we assume that this organism, first found by Söhngen, is mainly responsible for the terminal removal of acetate in methanogenic ecosystems. The name *Methanothrix söhngenii* was proposed.

All methane bacteria described up to now require in pure culture a pH for maximum growth in the range of about pH 7–8. This contradicts the many observations on methane formation in water logged, acidic peat bogs[25]. Russian investigators described a *Methanobacterium kuzneceovii* from such environments which reportedly grew at pH 4[26,27].

Types of methanogenic ecosystems

The methane generating ecosystems can be classified into two groups when their starting conditions are considered (fig.).

The chemolithotrophic ecosystems (pathway I in the figure) are the functionally most simple ones. Hydrogen and CO_2 from geochemical sources in terrestrial, sublacustrian or submarine thermal springs are exploited by chemolithotrophic methane bacteria according to reaction [1] for ATP synthesis. The energy conserved is used for CO_2-assimilation. Large populations of methane bacteria such as in Lake Kiwu (an African rift lake)[28] or in thermal springs in Yellowstone Park[17] characterize these ecosystems as typical sites of autotrophic production independant from photosynthesis. The methanogens function as pioneers in environments unexploitable by any other organism.

Two ecotypes must be discussed in group II of the figure, namely: 1. the rumen of cloven-footed wild or domestic animals and 2. natural or technical systems for anoxically degrading organic matter (organic muds, sediments, water saturated soils, sewage sludge or liquid waste digesters, 'biogas' installations, garbage dumps etc.). The educts for methanogenesis in these chemoorganotrophic, methanogenic systems are – in contrast to group I – fermentation products of organic matter of (mostly) biogenic origin. The primary substrates in rumen systems as well as in littoral swamps and muds are terrestrial or aquatic macrophytes containing roughly 35–45% cellulose and other polymeric carbohydrates, 20–30% lignin, 12–20% protein and 2–4% lipids. The sewage sludge fed to sludge digesters in municipal waste treatment plants is composed (on the average) of 10–15% cellulose and other structural carbohydrates, 6–7% lignin, 20–25% protein and 15–30% lipids. So-called 'biogas' plants at farms receive mostly animal excreta with about 14–25% cellulose, 8–15% lignin, 5–10% protein and 1.5–2.5% lipids (all figures on a dry weight basis). Low molecular weight dissolved compounds are present in negligible quantities in these natural substrates. In treatment systems for concentrated liquid wastes (mostly spent liquors from fermentation industries) sugars, short-chain fatty acids, amino acids and peptides represent the bulk of primary substrates.

Since the substrates in group II systems are in general insoluble polymers, the fermentation flora consists largely of species with exoenzymatic properties. Except for the rumen of domestic animals where extensive investigations on its species composition exist[29], the biocenosis responsible for the 'liquefaction' and fermentation in nature or in technical methane fermentations is still terra incognita. The common biochemical knowledge on pathways and products of fermentations gathered from pure strains of anaerobes, isolated from soils and muds, is insufficient for a mechanistic understanding of the dynamics of these complicated microbial communities. Although it may be assumed that many analogies exist to the rumen fermentation, a thorough microbiological investigation of sludge digesters and other methanogenic systems in nature would be gratifying.

Published data on the number and species of organisms active in the initial 'liquefaction/fermentation' of the primary substrates are to be considered with caution due to obvious methodological difficulties in 'counting' organisms (usually with MPN-methods) of highly different metabolic properties. Overall figures (excluding methane formers) in the order of magnitude of $2–3 \cdot 10^9$/ml were reported for the rumen[29,30]. In sludge digesters similar figures of $2–6 \cdot 10^9$/ml are indicated[31]. Siebert and Toerien[33] isolated more than 50 strains of proteolytic species of *Clostridium, Peptococcus, Eubacterium, Bacterioides* and others from digesting sludge. Their total number was estimated to be at least $6.5 \cdot 10^7$/ml, mostly strict anaerobes. The proportion of facultative anaerobic bacteria in digesters does not seem to exceed some 1% of the total count[31,32]. Anaerobic counts in the uppermost strata of the sediment in a hypertrophic lake amounted to $2–6 \cdot 10^6$ cells/g dry sediment (e.g. about $5–8 \cdot 10^5$ cells/ml) with 72% *Clostridia (C. bifermentans, C. sporogenes, C. butyricum)* and 5% *Eubacterium*[33]. Lignin decomposers have never been found although indications exist for the fermentation of some of the monomers to be expected from lignin break down[29,35]. In general, the relative concentration of lignin in the remains of a fermentation mixture increases (e.g. in sewage sludge from 9–10% up to about 17%[12]), showing that this important biogenic material is not noticeably attacked in methanogenic systems.

The fermentations of carbohydrates, proteins and fats yield the C_1 to C_4 monocarboxylic acids as the most oxidized terminal products plus H_2 and CO_2. Other compounds such as ethanol, lactate, succinate or

valerate have been found occasionally in insignificant amounts in fermentations well equilibrated around neutral pH. The main products are hydrogen and acetate. - The principle source of hydrogen is the phosphoroclastic reaction of pyruvate to acetylphosphate via acetyl-SCoA. The oxidant and hydrogen carrier in anaerobiosis is ferredoxin[36,37]. Further H_2 is released by formatelyase from formate produced by enterobacteria in the splitting of pyruvate to acetate and formate. The population density of these bacteria in sludge digesters and other anaerobic systems and, hence, the importance of this reaction, is not known. - A considerable part of the total of acetate formed is due to the splitting of pyruvate already mentioned above. Minor amounts of acetate may also be produced in this reaction sequence when reduced ferredoxin is reoxidized by CO_2 (homoacetate fermentation with e.g. *Clostridium formicoaceticum* and *Acetobacter woodii*). The oxydation of H_2 with CO_2 to acetate by *C. aceticum* may further contribute to the acetate pool, and the β-oxidation of higher fatty acids in substrates rich in fats (sewage sludge) will add appreciable amounts of acetate in such specific environments. The same pathway to acetylphosphates and ATP from other acyl-CoA esters (propionyl-SCoA, butyryl-SCoA etc.) yields probably most of the propionate and butyrate respectively. Insufficient knowledge of the species composition in sludges and mud makes it difficult to identify the specific metabolic reactions leading to the various products observed when, for some reason, their immediate further oxidation is inhibited. A last and pertinant source of both acetate and hydrogen is the final oxidation of ethanol, propionate and butyrate in non-rumen systems[39] which will be discussed below.

The analogy of the rumen and other methanogenic systems in group II of the figure ends at this point. In the rumen the organic acids and alcohols are absorbed by the rumen wall and transferred to the bloodstream of the animals, thus maintaining a favorable and well equilibrated environment for continuous fermentation, buffered at about neutrality by the rumen fluid. The hydrogen is respired to methane by the lithotrophic *M. ruminantium* at the same rate as it is formed.

In sediments, muds, sludge digesters etc. the terminal fermentation products cannot be eliminated as in the rumen and hence, would accumulate and acidify the environment if they were not further oxidized. Long experience shows indeed that large amounts of acetate, propionate, butyrate and other fatty acids accumulate at times in sludge digesters. This 'failing' of the fermentation process by acidification is accompanied by a notorious decrease in methane production and an increasing percentage of hydrogen in the digester gas. It can easily be demonstrated that acid accumulation and hydrogen pressure in the system are closely related[20,40,41]. This is obvious when the stoichiometry and the thermodynamics of the oxidation of propionate and butyrate is considered:

$$[3]\ CH_3CH_2COO^- + 2\,H_2O \leftrightarrows CH_3COO^- + 3H_2 + CO_2$$
$$\Delta G^{0'}\ (pH\ 7) = +81.6\ kJ/mole$$

$$[4]\ CH_3CH_2CH_2COO^- + 2H_2O \rightleftarrows 2CH_3COO^- + 2H_2 + H^+$$
$$\Delta G^{0'}\ (pH\ 7) = +41.6\ kJ/mole$$

The reactions become thermodynamically feasible ($\Delta G^0 \pm 0$ kJ/reaction) at reactant concentrations of 10^{-3} moles/l, pH 7 and 25 °C when the hydrogen pressure in the medium is held below 10^{-2} atm with butyrate or 10^{-4} atm with propionate respectively. For good growth of organisms using these acids as substrates, hydrogen pressures in the order of less than 10^{-5} atm must be maintained in the external medium when the oxidations should yield useful energy. The biochemistry of the oxidations [3] and [4] is not known. Protons are assumed to act directly as electron acceptors (hence the term 'obligate proton-reducing bacteria'[37]). However, neither the initial reaction with the acids nor the mechanism of electron disposal have been formulated as yet. Speculations on these reactions[36,42] cannot be substantiated unless pure cultures of the organisms involved are available.

The above thermodynamic considerations point to the fundamental difference between the rumen and the other methanogenic systems in nature: in the rumen the elimination of hydrogen by methanogenesis is only a facultative reaction. When the lithotrophic methanogens are absent after fasting of the animal or when they are suppressed by inhibitors, hydrogen instead of methane is eructated, apparently with no noticeable detriment to the feed utilization by the animal[29]. In the other methanogenic systems, however, oxidation of hydrogen by CO_2 to methane represents a reaction which decides on the existence or non-existence of anaerobic mineralization in nature, at least under conditions of low sulfate content of the environment. In regard to the problem of culturing the acid oxidizers, the analogy to the earlier experience with *M. omelianskii* which was found to be a 'syntrophic' association of a lithotrophic methane bacterium and an ethanol oxidizing, organotrophic microbe[15], is obvious. The anaerobic oxidation of ethanol is endergonic, although much less so than the oxidation of propionate or butyrate. The organism using ethanol (organism S) obviously found its ecological niche in close commensalism with the methane bacterium which provided for the low H_2 pressure, required for exergonic utilization of ethanol (so-called interspecies hydrogen transfer). It was but a logical consequence when Bryant's group tried to enrich acid oxidizers in co-culture with hydrogen consuming organisms. Reports of successful two-strain co-cultures of a propionate[43] or a butyrate oxidizer[44-46] with a

hydrogenotrophic methane bacterium or a sulfate reducer were recently published *(Syntrophobacter wolinii* and *Syntrophomonas wolfei* respectively). No culture technique has been found up to now for the separation of such 'syntrophic' pairs and getting the acid oxidizers in pure culture, because physico-chemical methods to absorb hydrogen from the medium at a rate, exceeding the rate of its production, have not been detected yet.

To sum up, it is now well established with these last findings that natural and technical methanogenic systems (except the rumen) require three groups of microbes (not two as previously thought) for accomplishing the transfer of organic matter to methane and CO_2, i.e. a) organisms for the fermentative oxidation of organics to the terminal products organic acids, H_2 and CO_2, b) organisms oxidizing the acids to acetate, H_2 and CO_2 and c) bacteria oxidizing H_2 with CO_2 and decarboxylating acetate to CH_4 and CO_2. It is further indispensable that the three groups form a biocenosis within the same ecological niche, to assert an environment equilibrated at a neutral pH. It is not amazing, therefore, that systems with large inputs of primary substrates (sludge digesters!) work best under continuous mixing (continuously stirred fermenters). For the same reasons, the many attempts to split sludge digestion into separated steps (e.g. 'liquefaction'/ fermentation within one compartment, methanogenesis within a second one) have never been successful.

Reactions competing with methane output in anaerobic ecosystems

Up to this point methanogenic systems low in sulfate, nitrite or nitrate have been considered. In marine environments and in many technical fermentations, however, these anions are present in the medium in appreciable concentrations and might affect methane output through 1. consumption of the educts for methanogenesis i.e. H_2 and/or acetate by organisms reducing sulfate or nitrogen oxides and 2. oxidation of methane within the system. The problem arose with observations in anaerobic marine sediments, indicating that dissolved methane in the interstitial water only appeared at depths where sulfate was nearly depleated[47,48]. Martens and Berner[49] concluded 'that sulfate reduction and methane production are mutually exclusive processes'. Since potentials of key redox reactions in desulfurication and methane formation do not sustain such an exclusion from a biochemical point of view, substrate competition must be considered as a mechanism. New isolations of desulfuricating bacteria from marine environments[50-52] which have considerably enlarged our knowledge on substrate requirements of this group, demonstrate the existence of chemoorganotrophic and chemolithotrophic species respiring H_2, propionate or butyrate and acetate with inorganic sulfur compounds as terminal electron acceptors and excreating H_2S. Others oxidize numerous organic, low molecular weight compounds to acetate, with again sulfur oxides as electron sink. Taking together the metabolic capacities of all presently known genera, the desulfuricating bacteria represent a group which is able to completely replace the biocenosis of methanogens and acid oxidizers for full 'mineralization' in an organotrophic, anaerobic ecosystem, when the supply of sulfate enables acceptance of all the electrons from the oxidation of the organic compounds introduced into the system. The higher metabolic versatility of this organism group as a whole, and the slightly more favorable thermodynamic situation in comparison to the restrictive biological conditions for methanogenesis (e.g. the obligate commensalism with H_2-oxidizers!) accounts for an obvious advantage of desulfuricating biocenoses over methanogenic associations. This does not exclude, however, simultaneous methane production and sulfate reduction in marine sediments as reported[53].

There remains the question of methane oxidation concurrent with methane production. In addition to studies on the possible impact of sulfate on methanogenesis, some evidence of the oxidation of methane in marine and fresh waters or sediments has been documented[54-57]. Since it seems well established that no organisms exist which oxidize methane anaerobically with NO_3^-, NO_2^- or SO_4^{2-} as terminal electron acceptors, some other mechanisms must be involved. Zehnder and Brock[58,59] observed that after injection of $^{14}CH_4$ into growing and methane producing pure cultures of a number of hydrogenotrophic methane bacteria, ^{14}C could be found in the culture vessels as $^{14}CO_2$ as well as in labelled biomass. With cultures of acetotrophic *M. barkeri* and *Methanothrix söhngenii* growing on acetate, the injected $^{14}CH_4$ produced labelled acetate, CO_2 and biomass. At low partial pressures of CH_4 and low rates of methanogenesis, the percentage of CH_4 'reconversion' was small ($< 1\%$). Increasing the pressure of $^{14}CH_4$ up to 20 atm in very actively digesting sewage sludge resulted, however, in losses of up to 90% of produced methane. The classical inhibitor of methanogenesis, 2 Br-ethanesulfonic acid in concentrations not to affect methanogenesis, inhibited methane oxidation to a great extent. This surprising reconversion of CH_4 to its educts is, of course, not a simple back reaction of the product formation. At present neither a satisfactory mechanism of this oxidation is known, nor is its ecological significance understood in regard to the functioning of a methanogenic system.

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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