PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Methanogenesis and Methanogenic Partnerships [and Discussion]

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Methanogenesis and methanogenic partnerships

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The microbial formation of methane from organic compounds greater than C_2 in chain length demands a mixture of methanogenic and chemoheterotrophic non-methanogenic bacteria. The chemoheterotrophic, non-methanogens initiate the reactions by well established pathways, hydrolysing complex polymers and fermenting the unit constituents to smaller end-product molecules, which are further metabolized by other chemoheterotrophs to acetic acid, H_2 and CO_2. The methanogens are essential physiological partners in the overall conversion of the initial substrates to this level because they oxidize H_2 and reduce CO_2 to form methane. The ultimate formation of acetate as the chief intermediate in this fermentation depends on the removal of H_2 by the methanogens. Otherwise, these acetogenic reactions are not thermodynamically feasible. Acetate, the major precursor of CH_4 in fermentation systems, is converted to CH_4 and CO_2 via a unique aceticlastic reaction. Examples of microbial partnerships producing methane from the fermentation of organic compounds are described.

Introduction

In a real sense, the methane-producing archaebacteria have always had a continuous historical and practical link to the study of microbial ecosystems and mixed culture communities. The pioneering of axenic culture by Koch and later investigators notwithstanding, the methanogens have been a group of microbes uniquely resistant to the 'pure culture' techniques of countless investigators. This culture dilemma has only recently yielded to the persistence of modern investigators with isolation of the first axenic methanogenic cultures by Schnellen in 1947. The reasons for this mixed culture history are based in the complex and intimate microbial interactions that are woven into the metabolic contract between the non-methanogenic, chemoheterotrophic and methanogenic bacteria. To study the methane fermentation is to study microbial ecology.

The environmental conditions under which methane is produced biologically are found in the organic sediments of most freshwater and marine environments as well as in the gastrointestinal tract of man and animals, particularly in the rumen of herbivores and the enlarged caecum of certain non-ruminants. In all of these habitats, the anaerobic conditions and the absence of light, NO_3^-, S^0, and SO_4^{2-} that accompany the presence of fermentable organic compounds lead to the biogenesis of methane. These physical and chemical selective pressures enrich for a specialized microbial population collaboratively engaged in the process of obtaining the most energy possible from organic compounds under anaerobic conditions. This is ultimately accomplished when the starting substrates are converted completely to CH_4 and CO_2. Of course, the actual metabolic events and the extent of the fermentation may be shaped by the specific conditions of the particular ecosystem. For example, in the gastrointestinal tract of animals, especially ruminants, the microbial conversion of organic compounds entirely to CH_4 and CO_2 is circumvented by the absorption of volatile acid fermentation products through the rumen

wall. Consequently, a significant part of the substrate carbon is used nutritionally by the animal host and less is converted to CH_4.

In certain aquatic systems, such as geothermal springs and Lake Kivu, one of the rift lakes of east-central Africa, abiogenic hydrogen may be an important substrate for reduction of CO_2 to CH_4, and organic substrates may contribute only a minor fraction to the total methane formed (Rudd & Taylor 1980). The impact of such an external H_2 source on the methanogenic conversion of organic compounds has not been evaluated. In marine or other aquatic sediments containing elemental sulphur or sulphate, sulphate-reducing bacteria may compete for H_2 as well as acetate.

This paper will primarily address the types of microbial partnerships in aquatic sediments and man-made anaerobic fermentations of organic compounds where the starting substrates are converted completely to CH_4 and CO_2. Whenever possible, the current binomial names of the methanogens proposed by Balch *et al.* (1979) are substituted for earlier names.

Brief review of early methanogenic studies

Unsuccessful attempts to culture methanogenic bacteria by Söhngen in 1906 led him to conclude that these organisms could not be obtained axenically, perhaps because of their 'slow growth rates or perculiarities of the organisms' (Söhngen 1906). Indeed, current knowledge confirms the slower growth rates of methanogens than most bacteria. Moreover, the biochemical and chemical peculiarities of this group mark them as distinct and separate from all other prokaryotic and eukaryotic cells (see later). In particular, two physiological properties of these organisms undoubtedly contributed heavily to the early culture difficulties: (1) the anaerobic nature of the methanogens, and (2) the limited range of methanogenic substrates. Although Söhngen recognized the first property by placing his cultures in an anaerobic desiccator evacuated with H_2 (Söhngen 1906), he was not successful in culturing colonies of these organisms probably because of inadequately low oxidation–reduction conditions. This technical problem was only solved much later by application of stricter anaerobic methods (Schnellen 1947; Stadtman & Barker 1951*b*) and eventually by application of the Hungate roll-tube technique (Smith & Hungate 1958). The nature of the second property, the range of methanogenic substrates, proved to be much more complex and its study is entangled in the history of the microbial ecology of the methane fermentation.

Studies on methanogens before Schnellen's work in 1947 all involved mixed or enrichment cultures in which physical and chemical selective pressures favoured growth of certain methanogens but did not exclude growth of other organisms. In spite of the lack of axenic cultures, methanogenic bacteria were identified in these enrichment cultures and named on the basis of morphological and presumed physiological properties. Some genera were even named without naming a species (Kluyver & Van Niel 1936). Early reports of cellulolytic methanogens were unconfirmed and gradually gave way to the hypothesis first proposed in 1890 by van Senus (as cited in Buswell & Hatfield (1939)) that methane was formed from the fermentation products of cellulose produced by non-methanogenic cellulolytic and associated bacteria. Some of the fermentation products included formate (Söhngen 1906), acetate (Hoppe-Seyler 1876) and H_2–CO_2 (Söhngen 1906) in addition to ethanol (Omeliansky 1916), propionate (Tarvin & Buswell 1934; Stadtman & Barker 1951) and butyrate (Söhngen 1906). Many other potential fermentation products, including longer chain volatile fatty acids and alcohols, were also

reported as methanogenic substrates, but the above products are the ones of chief interest in this paper because of their historical or actual importance, or both, in the methane fermentation.

Methanobacillus omelianskii

The idea that low molecular mass fermentation products were used as substrates by methanogens formed the basis for many attempts to isolate specific organisms on specific compounds. One of the first of these attempts was reported in 1916 by Omelianski who published photographs of an ethanol-oxidizing methanogen. This organism was later observed and named *Methanobacterium omelianskii* (*Methanobacillus omelianskii* (Barker 1956)) by Barker in 1936; in 1940 Barker reported its isolation. This culture, extant from that time, served as the focal starting point for developing our present understanding of the ecology of the methane fermentation.

The culturing of *M. omelianskii* was, in retrospect, one of the milestones in the history of methanogenesis because the choice of ethanol as substrate circumscribed the fate of the culture process and ordered the development of a co-culture: *M. omelianskii* was actually a monoxenic (two-membered) and not an axenic culture! This finding was reported in 1967 by Bryant *et al.* who demonstrated, in a series of carefully designed experiments, that the culture was composed of a chemoheterotrophic non-methanogen, the so-called S organism, and a methanogen, *Methanobacterium bryantii*, and each could be grown axenically. Moreover, they established the physiological interdependence of the two organisms when ethanol served as the energy source. Ethanol was oxidized by the chemoheterotrophic S organism to acetate and H_2, and H_2 was removed by reduction of added CO_2 to form methane by strain M.o.H., now called *Methanobacterium bryantii*. The S organism was apparently sensitive to its own H_2 end-product and furthermore could not oxidize ethanol in the presence of H_2; in turn, the methanogenic partner was dependent on the formed H_2 as an energy source since it could not use ethanol as a substrate. Hence the two organisms established a syntrophic mutualistically beneficial association in order to utilize ethanol. Neither isolate was capable of growing on ethanol by itself. This type of physiological interaction is characteristic of the methane fermentation and helps to explain some of the problems of earlier investigators in their attempts to isolate methanogens from the methanogenic conversion of substrates other than H_2–CO_2, formate, methanol, acetate and mono-, di- and trimethylamine.

Interspecies H_2 *transfer*

A physiological partnership between non-methanogenic chemoheterotrophs and methanogens was first predicted from ecological studies by Hungate (1966) of the rumen fermentation. Since axenic cultures of carbohydrate-using rumen bacteria often formed reduced fermentation products not found in the natural mixed rumen system, Hungate postulated that the electrons formed from the oxidation of fermentation substrates were used for the reduction of CO_2 to methane in the rumen. Hence in axenic cultures of chemoheterotrophs, interspecies electron disposal by methanogenic bacteria was unavailable, and formation of end-products by reduction of fermentation intermediates served as the final mechanism for electron disposal. In mixed culture systems, provided that the appropriate hydrogenase enzymes are present, the direction of fermentation-generated electrons is channelled towards proton reduction, i.e. H_2 formation, which becomes the main electron sink. This is only thermodynamically feasible (see Wolin (1976) for a discussion of the relation between ΔG and partial pressure of H_2) if the H_2 were

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

maintained at vanishingly low concentrations by removing it from the system, a task that is accomplished by methanogenic or other H_2-using non-methanogenic bacteria. This phenomenon, termed 'interspecies transfer of H_2' by Iannotti *et al.* (1973), was first systematically verified by using a chemostat co-culture of *Ruminococcus albus* and *Vibrio succinogenes* fermenting glucose (Iannotti *et al.* 1973). *R. albus*, growing alone, produced acetate, ethanol, H_2 and CO_2. In co-culture with *V. succinogenes* and added fumarate, which *R. albus* cannot metabolize, fumarate served as the terminal electron acceptor for *V. succinogenes* to form succinate, and the end-products of *R. albus* shifted completely to acetate and CO_2. Since *V. succinogenes* obtained energy by H_2 oxidation and fumarate reduction, these products must have resulted from an interspecies transfer of H_2, bypassing ethanol formation by *R. albus*, to form succinate by *V. succinogenes*. The final results of these reactions may be a higher cell yield, metabolism of a greater quantity of substrate by the co-culture, formation of a greater quantity of oxidized end-product, namely acetate, and greater net energy production (Iannotti *et al.* 1973; Thauer *et al.* 1977; Wolin 1976).

TABLE 1. THE ROLE OF METHANOGENS IN INTERSPECIES H_2 TRANSFER

non-methanogens only	*non-methanogens and methanogens*
organic compounds	organic compounds
↓	↓
↓ ⟶ $H_2 + CO_2$	↓ ⟶ $H_2 + CO_2$
↓	↓ ↓
volatile fatty acids, neutral end-products	↓ $CH_4 + H_2O$
	↓
	acetic acid
	↓
	$CH_4 + CO_2$

THE METHANOGENIC INTERACTION

The role of methanogens in interspecies H_2 transfer is illustrated in table 1. During the fermentation of organic substrates by chemoheterotrophs alone, the products are determined by the final organic electron acceptors that serve as the electron sink for the metabolic system. Hence, various types of organic end-products such as acetate, butyrate, propionate, ethanol, acetone, butanol and propanol may be produced. H_2 and CO_2 may also be common end-products of these reactions. However, in the presence of H_2-oxidizing methanogens, proton reduction becomes the major electron sink for the chemoheterotrophs and acetate is the major organic end-product: reduced end-products are formed at lower concentrations or not at all. If present, these reduced compounds may also be converted by methanogenic interspecies H_2 transfer to acetate by a second more specialized group of chemoheterotrophs (see later). The net result is the overall conversion of the starting substrates primarily to acetate, CH_4 and CO_2.

The fate of the acetate may differ from one ecosystem to another. In ruminants, acetate is absorbed through the rumen wall and most, if not all, of the methane is formed from H_2–CO_2. In marine and other aquatic systems where sulphate is present, sulphate reducers may compete for acetate and oxidize it to CO_2 and H_2O. However, in most methane-producing sediments or in anaerobic digesters, acetate is the chief precursor of methane (Jeris & McCarty 1965; Smith & Mah 1966) via a unique aceticlastic reaction in which the methyl group of acetate is converted directly to methane and the carboxyl group to carbon dioxide.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

In comparison with aerobic systems where oxygen serves as the final electron acceptor, very little of the substrate energy is made available for cell use during fermentative metabolism. For example, the standard free energy change for the oxidation of glucose by O_2 is -2.822 MJ mol^{-1}. Aerobic organisms may conserve approximately 45% of this available energy in the form of ATP by means of substrate-level and oxidative phosphorylation reactions. In contrast, anaerobic organisms obtain very little of this available substrate energy; most of it remains bound up in the fermentation products. In *Ruminococcus albus*, for instance, about 90% of the glucose energy still remains in the end-products of fermentation. By acetogenic and interspecies H_2 transfer reactions, these end-products may be converted entirely to acetate, H_2 and CO_2 and thence to CH_4 and CO_2, with no other fermentation products formed. When this occurs, an additional amount of the substrate energy is released. Nevertheless, 86% of the energy originally present in glucose still remains bound up in the end-product, CH_4! Thus, for a paltry increase in 4–5% of the available substrate energy, anaerobic chemoheterotrophic non-methanogenic and methanogenic bacteria have developed many types of partnerships in order to eke out a livelihood. The many combinations and permutations of these mutualistic associations form the basis of the methane fermentation. Tables 2–5 summarize examples of some specific relations that have been reported.

Low molecular mass compounds

The methanogenic metabolism of low molecular mass organic compounds other than formate, methanol, acetate and mono-, di- and trimethylamine is mediated by the joint efforts of proton-reducing, acetogenic chemoheterotrophs and H_2-oxidizing and aceticlastic methanogens. Thus, the older concept of the organism *Methanobacterium suboxydans* (Stadtman & Barker 1951*a*), which reportedly oxidized butyrate, valerate and caproate and formed methane by the CO_2 reduction route, has been replaced by the interspecies H_2-transfer concept. Likewise, the methanogenic fermentation of propionate by *Methanobacterium propionicum* or of ethanol, propanol, isopropanol, butanol, isobutanol or pentanol by other methanogens may also be explained by interspecies H_2-transfer.

Ethanol

In addition to the S organism (previously discussed), other anaerobic bacteria may also oxidize ethanol (table 2) provided that the reaction can be made thermodynamically favourable by linking proton removal to sulphate reduction or methane formation. This was accomplished for *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* by adding sulphate or by co-culturing each with *Methanobacterium bryantii* (M.o.H.) (Bryant *et al.* 1977). When a limiting amount of sulphate was added to an ethanol-using co-culture, the methane yield was reduced by an amount approximately equal to the reducing equivalents required for reduction of the added sulphate. If sulphate was added in excess, no methane was produced. It was clear that sulphate was a preferred electron acceptor over CO_2 reduction by interspecies H_2 transfer. However, if sulphate was exhausted, oxidation of ethanol could only continue via the methanogenic partnership. Chemoheterotrophs other than sulphate reducers may also engage in this type of mutualistic association as long as they produce the appropriate enzymes, for example *Thermoanaerobium brockii* may oxidize ethanol in partnership with *Methanobacterium thermoautotrophicum* (Ben-Bassat *et al.* 1981).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 2. Methanogenic interspecies H_2 transfer: obligate proton reduction

chemoheterotrophic H_2 producer	substrate	monoculture products	H_2-using methanogen	mixed culture products	reference
Thermoanaerobium brockii	ethanol	acetate (tr.), H_2 (tr.)	*Methanobacterium thermoautotrophicum*	acetate (↑), CH_4	Ben-Bassat *et al.* (1981)
S organism	ethanol	acetate (tr.), H_2 (tr.)	*M. bryantii*	acetate (↑), CH_4	Bryant *et al.* (1967) Reddy *et al.* (1972)
	other alcohols†	none	*Methanobrevibacter smithii*	corresponding acid‖, CH_4	Reddy *et al.* (1972)
	pyruvate‡§	ethanol, acetate, H_2	*M. smithii*	acetate (↑), CH_4	Reddy *et al.* (1972)
Desulfovibrio desulfuricans or *D. vulgaris*	ethanol	acetate (tr.), H_2 (tr.)	*M. bryantii*	acetate (↑), CH_4	Bryant *et al.* (1977)
	lactate	trace products	*M. bryantii*	acetate (↑), CH_4	Bryant *et al.* (1977)
	lactate	trace products	*Methanosarcina barkeri*	CH_4, CO_2	McInerney & Bryant (1981)
Selenomonas ruminantium	lactate§	acetate, propionate	*M. smithii*	acetate (↑), propionate (↓), CH_4	Chen & Wolin (1977)
Anaerovibrio lipolytica	lactate§	acetate, propionate, H_2 (tr.)	*M. ruminantium*	acetate (↑), propionate (↓), CH_4	Prins & Clarke (1980)

The fermentation products listed are the major products detected. CO_2, a major product in almost all cases, is not shown. Significant concentration changes in the mixed culture products are indicated as increases (↑) or decreases (↓). The disappearance of H_2 from the monoculture is accompanied by the appearance of CH_4 in the mixed culture.

† Other alcohols: *n*-propanol, *n*-butanol, isobutanol, *n*-pentanol. Acetaldehyde was also a substrate.

‡ Oxalacetate was also a fermentative substrate for S organism.

§ These substrates are metabolized fermentatively by axenic cultures of the chemoheterotroph shown; proton reduction is not obligate.

‖ Presumably the corresponding fatty acid was formed.

Other alcohols

The methanogenic metabolism of higher alcohols such as *n*-propanol, *n*-butanol, isobutanol and *n*-pentanol was also reported by Reddy *et al.* (1972) for the S-organism–methanogen co-culture. Growing axenically, the S organism produced little or no H_2 from these substrates, and consequently growth was very limited. Likewise, *M. smithii*, the methanogenic partner, will not grow on any energy source except formate and H_2–CO_2. However, in co-culture, good growth of both organisms was accompanied by production of large quantities of methane. The calculated H_2 equivalent based on methane produced was 25 times greater for *n*-pentanol and as much as 461 times greater for isobutanol than the measured amount of H_2 formed from these substrates by S organism alone. Utilization of isopropanol and secondary butanol by S organism or the co-culture could not be shown. These findings lead to the conclusion that methane formation from historically important methanogenic substrates (see Barker 1956), including all alcohols higher than methanol and all volatile acids higher than acetate, may conform to the general picture of interspecies H_2 transfer (Bryant *et al.* 1967).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Pyruvic acid

When pyruvate served as substrate for S organism growing alone, it was readily converted to ethanol, acetate and CO_2 with H_2 present in trace amounts (Reddy *et al.* 1972). However, in co-culture with *Methanobrevibacter smithii*, acetate, CH_4 and CO_2 became the major end-products and ethanol was present in trace amounts. The increased acetate was proportional to decreased ethanol and the quantity of CH_4 formed was stoichiometrically related to the quantity of reducing equivalents diverted away from ethanol formation. No H_2 was detected because it was oxidized by CO_2 reduction to methane. Thus, in the presence of a H_2-oxidizing methanogen, the electron flow from pyruvate dissimilation was directed toward proton reduction; consequently, the formation of CH_4 and not ethanol was the final reaction for electron disposal, and acetate, a more oxidized compound than ethanol, was the end-product (see table 2). In addition to this shift in end-product composition from a more reduced to a more oxidized form, the co-culture of S organism and *M. smithii* also utilized more pyruvate substrate. Consequently, a larger mass of bacteria and possibly also a greater net energy conservation may result from acetyl-phosphate conversion to acetate instead of acetaldehyde reduction to ethanol.

Lactic acid

A co-culture on lactate of either *Desulfovibrio desulfuricans* or *Desulfovibrio vulgaris* with *Methanobrevibacter bryantii* yielded only acetate, methane and CO_2 as products (Bryant *et al.* 1977). Addition of sulphate shifted the flow of electrons toward sulphate reduction, with formation of acetate, CO_2 and sulphide but not methane. This lack of methanogenesis may be due to the thermodynamically favoured reduction of sulphate or perhaps to the proximity of appropriate oxidation–reduction systems within one organism, thus obviating the transfer of H_2 between organisms. However, in the absence of sulphate, interspecies H_2 transfer by methanogenic bacteria is the only means for obtaining energy from lactate and the desulfovibrio then become acetogenic chemoheterotrophs with proton reduction as their only means of electron disposal. A coculture of *Methanosarcina barkeri* and *D. desulfuricans* or *D. vulgaris* also metabolized lactate to acetate, CH_4 and CO_2 (McInerney & Bryant 1981), but the acetate was then further converted to CH_4 and CO_2 by the aceticlastic *M. barkeri*. Acetate was not metabolized until the preferred methanogenic substrate, H_2–CO_2, was exhausted. This finding agreed with axenic culture studies of *M. barkeri* (Mah *et al.* 1981; Smith & Mah 1980) and implied some type of regulatory action governing utilization of substrate. The final result of this reaction was the complete conversion of lactate to CH_4 and CO_2.

In *Selenomonas ruminantium*, an anaerobe that is not sulphate-reducing, lactate was fermented to propionate, acetate and CO_2. Inoculation of *S. ruminantium* into a pre-grown culture of the methanogen *Methanobrevibacter smithii* resulted in a shift of these fermentation products to more acetate and less propionate, which accompanied CH_4 formation by the methanogen (Chen & Wolin 1977; Scheifinger *et al.* 1975). However, no increase in overall production of electrons, as calculated from CH_4 formation, appeared to occur in this latter couplet, implying that the utilization of lactate was not dependent on interspecies H_2 removal.

Propionic and butyric acids

The methanogenic metabolism of propionate and butyrate in enrichment cultures and domestic sludge was thoroughly investigated by Smith and his collaborators (1980) who

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

demonstrated the formation of H_2 during fermentation of both propionate and butyrate. Smith & Shuba (1973) stripped H_2 from a propionate fermentation by vigorously sparging the system with CO_2, which was absorbed in an alkaline solution after collection. After absorption, the remaining gas phase was diluted in N_2 and analysed for H_2. At a sparge rate of *ca.* 950 ml min^{-1}, 10 ml H_2 per 500 ml culture was produced hourly from a propionate enrichment; no H_2 was detected without added propionate nor in acetate enrichments. This finding showed unequivocally that significant quantities of H_2 were evolved during propionate metabolism, though no H_2 was detectable during non-sparged conditions because of its rapid oxidation to CH_4. Similar findings were obtained with butyrate as substrate. When either the propionate or butyrate fermenting system was sparged with H_2, methanogenesis from these substrates was completely inhibited. This indicated that the oxidation of propionate or butyrate was probably dependent on H_2 removal, as might be expected during interspecies H_2 transfer. Further evidence for this role was shown by the large numbers of H_2-oxidizing methanogens present in these enrichments and by the fact that vigorously sparged H_2–CO_2 is used rapidly without a lag (Smith 1980; unpublished findings of Bryant as cited in McInerney & Bryant (1980)). The catabolism of propionate or butyrate is much less favourable thermodynamically than either ethanol or lactate (McInerney & Bryant 1980):

	$\Delta G^{\circ\prime}$ for reaction † / kJ
1. $CH_3CH_2COO^- + 3H_2O \longrightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$;	+76.1
2. $CH_3CH_2CH_2COO^- + 2H_2O \longrightarrow 2CH_3COO^- + H^+ + 2H_2$;	+48.1
3. $CH_3CH_2OH + H_2O \longrightarrow CH_3COO^- + H^+ + 2H_2$;	+ 9.6
4. $2CH_3CHOHCOO^- + 4H_2O \longrightarrow 2CH_3COO^- + 2HCO_3^- + 2H^+ + 4H_2$	+ 9.2

† Taken from Thauer *et al.* (1977).

H_2 formed from the oxidation of propionate or butyrate comes from electrons generated at a much higher potential than ethanol or lactate (see table 5 in Thauer *et al.* (1977)). Thus if these reactions are to be thermodynamically favourable, the partial pressure of H_2 must be maintained at even lower levels for propionate (10^{-5} atm†) and butyrate (10^{-6} atm) than ethanol (10^{-3} atm) and lactate (10^{-3} atm) at substrate concentrations of 10^{-4} M (McInerney & Bryant 1980). The actual existence of propionate and butyrate-oxidizing, acetogenic, obligate proton-reducing bacteria was verified by growth of the following co-cultures reported by Bryant and his collaborators of two different organisms, each capable of metabolizing one of these substrates (table 3). Isolation in axenic culture of either chemoheterotroph was not possible because of the absolute and inescapable requirement for the H_2-transfer reaction.

Butyrate oxidizer

The first co-culture of an obligate proton-reducing acetogenic bacterium was the butyrate-oxidizing organism reported by McInerney *et al.* (1979). This organism, initially co-cultured with butyrate as a substrate, the H_2-using *Desulfovibrio* G11 as a syntrophic partner, and sulphate as a terminal electron acceptor, was named *Syntrophomonas wolfei* (McInerney *et al.* 1981). Its co-culture confirmed the hypothesis (Bryant *et al.* 1967; Bryant 1976) that proton-reducing, acetogenic bacteria were responsible for the oxidation of traditional methanogenic fatty acids greater than two carbons in length. The carefully considered choice of a H_2-using

† 1 atm = 101 325 Pa.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 3. METHANOGENIC INTERSPECIES H_2 TRANSFER: ACETOGENIC OBLIGATE PROTON REDUCERS

chemoheterotrophic H_2 producer	substrate	monoculture products	H_2-using bacterium	mixed culture products	reference
Syntrophomonas wolfei	butyrate	none	*Methanospirillum hungatei*†	acetate, CH_4	McInerney *et al.* (1979)
	valerate	none	*M. hungatei*	acetate, propionate, CH_4	McInerney *et al.* (1981)
Syntrophobacter wolinii	propionate	none	*Desulfovibrio* sp. + sulphate	acetate, H_2S	Boone & Bryant (1980)
	propionate	none	*Desulfovibrio* sp. + *M. hungatei*	acetate, CH_4	Boone & Bryant (1980)

† *Methanobacterium bryantii* and *Methanobrevibacter arboriphilus* were also tested but did not support growth comparable with *M. hungatei*.

sulphate reducer as the syntrophic partner made the oxidation of butyrate thermodynamically more favourable than using a methanogen since the oxidation of H_2 by sulphate reduction is thermodynamically more favourable than CO_2 reduction. Once *S. wolfei* was obtained in co-culture, the sulphate reducer could be replaced by the methanogen *Methanospirillum hungatei*, which predominated in the butyrate enrichment used as an inoculum. Although the first successful co-culture of *S. wolfei* was facilitated by selection of a sulphate-reducing partner, the methanogen *M. hungatei* was used in subsequent direct isolations of another strain of *S. wolfei* from a different sewage-inoculated butyrate enrichment (McInerney *et al.* 1979). However, neither *M. bryantii* nor *M. arboriphilus* was capable of supporting equivalent growth in co-culture with *S. wolfei*, indicating differences in the syntrophic requirements provided by the methanogen. The generation times were reported only for the co-cultures of *S. wolfei* – *Desulfobivrio* and *S. wolfei* – *M. hungatei*; they were 54 h and 84 h respectively. The shorter generation time of the sulphate-reducer couplet agrees with the more favourable free energy of the sulphate-reducing reaction involved. Perhaps the diminished growth of the *M. bryantii* or *M. arboriphilus* couplets (compared with *M. hungatei*) may be related to differences in the K_m for H_2 required by these methanogens. Such values have not been reported.

The assiduous studies of McInerney *et al.* (1979, 1980, 1981) have led to the co-culture and description of four strains of *S. wolfei* from two different parts of the world: from Göttingen, F.R.G., and from Urbana, Illinois, U.S.A. The ubiquity of *S. wolfei* is further illustrated by its isolation from butyrate enrichments initiated from several primary habitats including anaerobic digester sludges (Göttingen and Urbana), creek sediment (Urbana) and bovine rumen fluid (Urbana), and from its observation in butyrate enrichments from two different lagoons (Urbana). *S. wolfei* obtains energy from the anaerobic β-oxidation of the straight chain monocarboxylic saturated C_4 to C_8 fatty acids and isoheptanoate in co-culture with a H_2-using partner. It does so by oxidizing the even-chained fatty acids exclusively to acetate and the odd-chained acids to propionate and acetate with proton reduction as its only means of electron disposal. Isoheptanoate is degraded to isovalerate, acetate and H_2. Potential substrates, including other branched-chain and long-chain fatty acids, methanol, ethanol and higher alcohols, sugars, amino acids, intermediates of glycolysis and the Krebs cycle, yeast extract, proteose peptone, or trypticase, did not support growth of the co-cultures. Likewise, the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

methanogenic partner could not be replaced by any of 15 chemical electron acceptors tested (McInerney & Bryant 1980; McInerney *et al.* 1979, 1981) with butyrate as the oxidizable substrate. Butyrate oxidation is completely inhibited by an initial partial pressure of 0.8 atm H_2 in co-culture with *M. hungatei* in agreement with the findings of Smith (Smith 1980) on mixed-culture enrichment systems.

S. wolfei is a Gram-negative, non-sporeforming, slightly helical rod, 0.5–1.0 by 2.0–7.0 μm with slightly tapered rounded ends. Most cells occur singly or in pairs with helical chains of three or more. PHB is present. Cells possess from two to eight flagella inserted laterally in a single row on the concave side. Sluggish twitching motility was observed. The presence of muramic and mesodiaminopimelic acid confirms the peptidoglycan nature of the unusual multilayered cell wall. The organisms are sensitive to penicillin.

Propionate oxidizer

The ubiquitous sulphate-reducing bacteria have also been implicated in the oxidation of propionate and may account for part of the propionate oxidation in anaerobic systems, provided that sulphate is present. The types of sulphate-reducing species involved appear to differ from the usual vibrio morphology and belong to new, as yet undescribed, genera (Bryant, personal communication, and Widdel & Pfennig as cited by McInerney & Bryant (1980)). A propionate-oxidizing sulphate reducer, probably *Desulfobulbous*, was present in low numbers (100–1000 nl inoculum) in the roll-tube dilution experiments of Boone & Bryant (1980). This organism presumably oxidized propionate to acetate and CO_2 by sulphate reduction to sulphide. However, upon extended incubation of the same dilution series for 6 weeks, large (> 1 mm), isolated, dark-centred colonies were visible at dilutions receiving only 10 and 1 nl of inoculum, indicating their presence in higher numbers than the sulphate reducer. Such colonies were present only when a lawn of *Desulfovibrio* sp. was introduced to mediate interspecies H_2-transfer from the propionate-oxidizing proton reducer to the H_2-oxidizing sulphate reducer. In the absence of propionate, no such dark-centred colonies formed. These colonies yielded an organism named *Syntrophobacter wolinii*, a refractile Gram-negative, non-sporeforming rod that occurred singly, in pairs, short chains, or long, sometimes irregular, filaments (Boone & Bryant 1980). It could only be grown in monoxenic co-culture with the sulphate reducer in the presence of sulphate. Only propionate was oxidized; acetate, butyrate, caproate and palmitate were not. Attempts to substitute the methanogen *Methanospirillum hungatei* for *Desulfovibrio* were unsuccessful. Even in the prolonged absence of sulphate (Boone, personal communication), *Desulfovibrio* remained, albeit at low numbers, in the co-culture of *S. wolinii* and *M. hungatei*. However, under these conditions, propionate was metabolized to acetate, CH_4 and CO_2. The inability to co-culture *S. wolinii* and *M. hungatei* without *Desulfovibrio* was not resolved. The generation time of the dixenic co-culture of *S. wolinii*, methanogen, and sulphate reducer was about half as fast (161 ± 18 h) as the monoxenic sulphate-containing culture of *S. wolinii* and *Desulfovibrio* (87 ± 7 h) (Boone & Bryant 1980). Although the choice of *Desulfovibrio* as a syntrophic partner was based on the thermodynamic advantage of sulphate over CO_2 as a terminal electron acceptor, it appeared that some other, as yet unexplained, factor(s) may be involved in co-culturing the propionate oxidizer at the monoxenic level with a methanogenic partner. Nevertheless, it was clear from these experiments that propionate-oxidizing acetogenic organisms other than sulphate reducers do exist and may be linked to methanogenesis in Nature. The relationship is, in fact, too intimate to yield to current culture attempts at the axenic level.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Carbohydrates

Because of increasing interest in methane production, attention has focused on the use of known two-membered (monoxenic) cultures to convert cellulose or free sugars to methane and other end-products. In all of these couplets (tables 4 and 5), the carbohydrates are converted to a more oxidized state (more acetate) in the presence of the methanogenic partner. Only when the selected methanogen was *M. barkeri* was there degradation of the starting free sugar (Winter & Wolfe 1980) or cellulose (Laube & Martin 1981) completely to CH_4 and CO_2 because of the aceticlastic reaction mediated by this methanogen. Obligate proton reduction was not important for the fermentative chemoheterotroph because it had several routes for terminal electron disposal.

Table 4. Methanogenic interspecies H_2 transfer: fermentative H_2 production from sugars

chemoheterotrophic H_2 producer	substrate	monoculture products	H_2-using methanogen	mixed culture products	reference
Eubacterium cellulosolvens	cellobiose	butyrate, lactate, H_2	*Methanobrevibacter ruminantium*	acetate (↑), butyrate (↓), lactate (↓), CH_4	Prins & van den Vorstenbosch (1975)
Selenomonas ruminantium	glucose	lactate, acetate, propionate	*Methanobrevibacter smithii*	acetate (↑), lactate (↓), propionate CH_4	Chen & Wolin (1977), Scheifinger *et al.* (1975)
Clostridium cellobioparum	glucose	acetate, formate, ethanol, H_2	*M. smithii*	acetate (↑), ethanol (↓), formate (↓), CH_4	Chung (1976)
Thermoanaerobium brockii	glucose	acetate, ethanol, lactate, H_2	*Methanobacterium thermoautotrophicum*	acetate (↑), ethanol (↓), lactate (↓), CH_4	Ben-Bassat *et al.* (1981)
Anaeroplasma sp.	glucose	butyrate, propionate, H_2	*Methanoplasma elizabethii*	acetate (↑), butyrate (↓), propionate (↓), CH_4	Rose & Pirt (1981)
Acetobacterium woodii	fructose	acetate	*Methanobrevibacter arboriphilus* AZ†	acetate (↓), CH_4	Winter & Wolfe (1980)

The fermentation products listed are the major products detected. CO_2, a major product in almost all cases, is not shown. Significant concentration changes in the mixed culture products are indicated as increases (↑) or decreases (↓). The disappearance of H_2 from the monoculture is accompanied by the appearance of CH_4 in the mixed culture.

† *Methanobacterium formicicum*, *Methanobacterium bryantii*, *M. bryantii* M.o.H.G., and *Methanosarcina barkeri* gave similar results when substituted for *M. aboriphilus* AZ.

Free sugars

Cellobiose, glucose and fructose have been reported as substrates for six fermentative chemoheterotrophic and methanogenic couplets (table 4). In axenic culture, *Clostridium cellobioparum* fermented glucose to ethanol, formate, acetate and H_2. In the presence of *M. ruminantium*, these products shifted quantitatively to more acetate and less ethanol and formate

(Chung 1976). H_2 was no longer detectable since it was oxidized at the expense of CO_2 reduction to methane. Palladium black could be substituted for the methanogen to remove H_2 from the axenic culture; in this case the end-products were similarly more oxidized, though, of course, CH_4 was not formed.

Even when acetate was not present as an axenic end-product, it still was the predominant oxidized product in co-culture with the methanogen. A shift in products was typical in every case regardless of the type of fermentation pathway or products formed in axenic culture. According to a recent report, an anaerobic chemoheterotrophic mycoplasma (*Anaeroplasma* sp.) coupled to a methanogenic wall-less organism (*Methanoplasma elizabethii*) also fermented glucose in this manner (Rose & Pirt 1981). Thus the oxidation of H_2 by the methanogen regulated the flow of electrons and affected the products formed. Both mesophilic and thermophilic couplets responded similarly.

In the experiments with *Acetobacterium woodii* coupled to *Methanobacterium arboriphilus* strain AZ, *M. bryantii*, *M. bryantii* strain M.o.H.G. or *M. formicicum*, fermentation of either glucose or fructose gave rise only to acetate with or without the methanogen (Winter & Wolfe 1980). In fact, with the methanogen, less acetate was formed because H_2 was channelled away from CO_2 reduction to acetate by *Acetobacterium woodii* to methane formation by the methanogen. *A. woodii*, growing axenically, produced 3 mol acetate per mole of glucose, presumably via mixotrophic acetate formation from glucose and metabolically produced H_2–CO_2 (Braun & Gottschalk 1981). When coupled to a strain of *M. barkeri* adapted to acetate utilization, fructose or glucose was completely converted to CH_4 and CO_2 in chemostat culture (Winter & Wolfe 1979). In this case, a food chain effect rather than interspecies H_2 transfer was more probably responsible for conversion because H_2 was first used to reduce CO_2 to acetate without competition from the methanogen; only then was acetate entirely converted to CH_4 and CO_2 by *M. barkeri*.

Cellulose

The fermentation of cellulose by chemoheterotrophic and methanogenic couplets again produced the same type of shift in end-products in axenic compared with monoxenic co-cultures (table 5). The thermophilic co-culture of *Clostridium thermocellum* and *M. thermoautotrophicum* exhibited faster growth rates (but no significant increase in yield) on cellulose than an axenic culture of the cellulolytic organism alone (Weimer & Zeikus 1977). A dixenic co-culture of *Acetivibrio cellulolyticus* with *Desulfovibrio* sp. and *M. barkeri* resulted in the complete degradation of cellulose to CH_4 and CO_2 in 7 days (Laube & Martin 1981). *A. cellulolyticus* played the role of fermentative chemoheterotroph by metabolizing the cellulose to ethanol, acetate, H_2 and CO_2. The *Desulfovibrio* then oxidized the ethanol to acetate by an obligate proton reduction linked to the methanogen, which oxidized H_2 and reduced CO_2 to CH_4. Acetate was then dissimilated to CH_4 and CO_2 by *M. barkeri*. A general interspecies H_2 transfer was implied by the slight increase (compared with the axenic culture) in oxidized end-products in the co-culture controls coupling either *A. cellulolyticus* – *Desulfovibrio* or *A. cellulolyticus* – *M. barkeri*. An active interspecies H_2 transfer at this level might have yielded a much greater proportion of oxidized to reduced product. Ethanol oxidation via obligate proton reduction, which demands interspecies H_2 transfer, was demonstrated between *Desulfovibrio* and the methanogen. Thus this dixenic co-culture exhibited the types of H_2-transfer reactions and syntrophic relations predicted for mixed-culture fermentations with unknown numbers and types of bacteria. Since

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

only batch cultures of unknown proportions of species were examined and concentrations of end-products were not reported for all experiments, any significance due to a food chain effect could not be evaluated.

The transfer of H_2 between two species may range from the mandatory relation established between an obligate proton reducer and a H_2-oxidizing partner to the general relation between any fermentative H_2-producer and any H_2-oxidizing organism. In the complete conversion of complex organic compounds to CH_4 and CO_2, both extremes of relations are important. *S. wolfei* and *S. wolinii* are examples of acetogenic, obligate proton reducers which have an absolute dependence on removal of H_2 by interspecies H_2 transfer and occupy a very narrow metabolic and ecologic niche by mediating thermodynamically less favoured reactions than other chemoheterotrophs. These reactions are not, *sensu stricto*, fermentative since proton reduction, and not reduction of some organic compound, is the final electron acceptor reaction. Without these organisms, reduced fermentation products such as propionate and butyrate may accumulate and methane formation diminished.

TABLE 5. METHANOGENIC INTERSPECIES H_2 TRANSFER: FERMENTATIVE H_2 PRODUCTION FROM CELLULOSE

chemoheterotrophic H_2 producer	substrate	monoculture products	H_2-using methanogen	mixed culture products	reference
Ruminococcus flavefaciens	cellulose	acetate, succinate, formate, H_2	*Methanobrevibacter smithii*	acetate (↑), succinate (↓), CH_4	Latham & Wolin (1977)
Clostridium thermocellum	cellulose	acetate, butyrate, ethanol, lactate, H_2	*Methanobacterium thermoautrotrophicum*	acetate (↑), butyrate, lactate, ethanol (↓), CH_4	Weimer & Zeikus (1977)
Acetivibrio cellulolyticus	cellulose	acetate, ethanol, H_2	*Methanosarcina barkeri* +/− *Desulfovibrio* sp.	acetate (↑), ethanol (↓), CH_4	Laube & Martin (1981)

The fermentation products listed are the major products detected. CO_2, a major product in almost all cases, is not shown. Significant concentration changes in the mixed culture products are indicated as increases (↑) or decreases (↓). The disappearance of H_2 from the monoculture is accompanied by the appearance of CH_4 in the mixed culture.

Chemoheterotrophic fermentative bacteria are involved in interspecies transfer of H_2 of a less dependent nature. In this case, the normal fermentative pathways may lead to proton reduction as one of several choices for electron disposal. If protons are removed by H_2-oxidizing methanogens, the electron flow is diverted and the fermentation products shift from a more reduced to a more oxidized form (tables 4 and 5). Such a shift in products is only exhibited by fermentative chemoheterotrophs capable of metabolizing the starting substrate without assistance from any other organism. Strict obligate acetogenic proton reducers do not exhibit such a shift in oxidation state of the products (table 3). When grown axenically, they either produce trace quantities of acetate and H_2 or nothing at all. When S organism or *Thermoanaerobium brockii* (table 2) metabolizes ethanol, it does so according to the strictest definition of obligate acetogenic proton reduction coupled to H_2 transfer. However, when S organism metabolizes pyruvate (table 2), or *T. brockii* metabolizes glucose (table 4), they do so fermentatively and in

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

axenic culture. Thus both S organism and *T. brockii* have the option of switching to a fermentative pathway given the appropriate substrate. This is not true of *S. wolfei* or *S. wolinii*, which are restricted to obligate proton reduction coupled to interspecies H_2 transfer. These organisms thus represent a unique group of bacteria that occupy an ecological niche that is apparently not available to any other group of organisms. The sulphate-reducing bacteria of Widdel & Pfennig (personal communication from Bryant), which may utilize volatile fatty acids and alcohols as well as aromatic compounds, have not been successfully grown by substituting a H_2-oxidizing methanogen for sulphate and hence may not compete for this niche.

The methanogenic archaebacteria

As a group, the methanogens are related physiologically, biochemically and at the molecular level. For energy generation, they are restricted to the production of methane from simple organic compounds, probably none greater than two or perhaps three carbons in length. Their known substrates are H_2–CO_2, formate, methanol, acetate, and mono-, di- and trimethylamine. CO may also be converted to CH_4 (Daniels *et al.* 1977; Kluyver & Schnellen 1947) but it is not an important substrate. All are strict anaerobes and require an oxidation–reduction potential of -330 mV (Smith & Hungate 1958) or less for methanogenesis. They contain or require the unique coenzyme M ($HS\text{-}SCH_2CH_2SO_3^-$) (McBride & Wolfe 1971; Taylor *et al.* 1974) and F_{420} in addition to several other less well described cofactors (Wolfe 1979). Nickel, as well as cobalt and molybdenum, are required for the growth of at least one methanogen (Schönheit *et al.* 1979). The 16*S* rRNA catalogues of the methanogens resemble each other but are distant from both eukaryotes and other prokaryotes (Balch *et al.* 1979; Fox *et al.* 1977). Based on analyses of these nucleotide sequences, Balch *et al.* (1979) proposed a taxonomic reorganization of the methanogens. As in other archaebacteria, the characteristic tRNA of methanogens differs from prokaryotes and eukaryotes; all species lack ribothymidine and 7-methylguanosine (Gupta & Woese 1980). The cell walls do not contain peptidoglycan or D-amino acids. Methanogens with a pseudomurein wall structure exhibit individual differences mainly in sugar structure or content, but amino acid substitutions may also occur (Kandler & König 1978). Some methanogens may have cell walls made up primarily of protein subunits and others of heteropolysaccharides. The antigenic map of 17 methanogenic isolates representative of the spectrum of existing axenic cultures (not including 1981 isolates) was determined by multiple assay with antibody probes (Conway de Marcario *et al.* 1981). Antigenically related strains coincided with the proposed classification of Balch *et al.* (1979) and also uncovered new relations.

Ether-linked polyisoprenoid lipids are the predominant lipid component (Balch *et al.* 1979). The polar lipids composed 70–80% of the total whole cell lipids and are principally C_{20} phytanyl and C_{40} biphytanyl glycerol ethers, depending on the genus (Tournabene & Langworthy 1978). The neutral lipids, which accounted for the remaining 20–30%, comprised a wide range (C_{14} to C_{30}) of isoprenoid hydrocarbons, with squalene as a common major component (Tournabene *et al.* 1979). The mechanism of ATP synthesis is not known; quinones may not be present (R. K. Thauer, as cited in Wolfe (1979)), although cytochrome b_{559} has been reported in *M. barkeri* (Kühn *et al.* 1979). The path of carbon assimilation, and especially CO_2 fixation, although under study (Fuchs & Stupperich 1980), has not yet been defined.

There are now 9 genera and 17 species of methanogens reported in axenic culture from many different habitats, including an 85 °C Icelandic volcanic spring where an extreme thermophile,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Methanothermus fervidus (temperature range 65–97 °C, optimum 83 °C) was recently isolated (Stetter *et al.* 1981). Geothermal H_2–CO_2 apparently supported growth in Nature. Also, the recent report of *Methanoplasma elizabethii* (Rose & Pirt 1981) extends the methanogens into the wall-less forms of archaebacteria.

Summary

In summary, there appear to be three distinct groups of bacteria (Bryant 1976) that participate in the decomposition of complex organic compounds completely to CH_4 and CO_2.

1. The chemoheterotrophic fermentative H_2-producers, which may hydrolyse complex polysaccharides, proteins and other macromolecules to their unit constituents and further degrade them to fermentation products, including neutral compounds, volatile fatty acids, H_2 and CO_2. Some chemoheterotrophic H_2-producers may also ferment low molecular mass end-products such as pyruvate and lactate.

2. The chemoheterotrophic non-fermentative acetogenic obligate proton reducers, which oxidize fatty acids three or more carbons in chain length and alcohols two or more carbons in chain length, producing acetate, H_2, and some CO_2 as end-products. These organisms absolutely depend on interspecies H_2-transfer reactions.

3. The methanogens, which perform two principal metabolic roles in mixed culture fermentation systems: (i) they maintain electron flow toward proton reduction by means of H_2 oxidation and CO_2 reduction to form CH_4 thereby creating a shift in reduced to oxidized end-products produced by the chemoheterotrophic fermentative bacteria as well as supporting the acetogenic obligate proton reducers; (ii) they remove the acetate end-product generated by the interspecies H_2-transfer reactions by an aceticlastic reaction to form CH_4 and CO_2.

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Discussion

J. G. Kuenen (*Microbiology Department, Delft University of Technology, The Netherlands*). There is no doubt that interspecies hydrogen transfer is of major importance in the growth of organisms on compounds like ethanol and propionate. But in the same reaction producing H_2, acetate is also produced. Removal of acetate from this reaction should also be very important in driving the decomposition of ethanol or propionate. Does Professor Mah agree that we also should pay more attention to *acetate* removal and not put all our emphasis on hydrogen removal?

R. A. Mah. Removal of all end-products, including acetate, would, of course, benefit the decomposition of ethanol, propionate, etc. In fact, Ferry & Wolfe (1976) calculated the $\Delta G^{\circ\prime}$ for methanogenic conversion of benzoate via intermediate formation of H_2, acetate and formate if these products were not removed and showed that product removal was necessary for optimal

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

conversion of benzoate. However, removal of acetate is not a significant driving force for substrate utilization. Externally added H_2 completely inhibited the conversion of ethanol, propionate, butyrate, etc. (Bryant *et al.* 1967; Smith 1980) enrichment cultures, yet added or increased acetate had no such effect. However, I do agree that we should pay more attention to acetate removal for the primary reason that acetate is the direct precursor of 60% or more of the methane in all digester systems. We have, in fact, concentrated most of our laboratory efforts on aceticlastic organisms.

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Ferry, J. G. & Wolfe, R. S. 1976 Anaerobic degradation of benzoate to methane by a microbial consortium. *Arch. Microbiol.* **107**, 33–40.

J. R. Quayle, F.R.S. (*Department of Microbiology, University of Sheffield, U.K.*). The first question is whether any factors have been identified that explain why some methanogenic bacteria but not others make effective partners for removing hydrogen from the obligate proton reducers.

The second question is whether any fortuitous metabolism of methyl CoM reductase has been detected. This might happen by formation, from some constituent of the growth medium, of an analogue of methyl CoM that was then reduced to a compound other than methane.

R. A. Mah. In reply to the first point, although it seems reasonable that some nutritional factor may be required to establish an appropriate syntrophic relation for interspecies H_2 transfer, no direct evidence of this has been reported. The effectiveness of one methanogen over another may instead be related to its K_m for H_2; thus *Methanospirillum hungatei* may have a much greater ability to use lower concentrations of H_2 than either *Methanobacterium bryantii* or *Methanobrevibacter arboriphilus* and hence is more successful in coupling with *Syntrophomonas wolfei*. However, the K_m for H_2 has not yet been reported for these methanogens.

Evidence supporting the type of reaction mentioned in the second question was reported by Gunsalus *et al.* (1978), who showed that ethylthiosulphonic acid (ethyl-S-CoM) was converted by methyl CoM reductase of cell-free extracts from *Methanobacterium thermoautotrophicum*. Recent ecological evidence from the estuarine environment (Oremland 1981) showed that ethane may be similarly formed by sediment slurries and enrichment cultures.

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