ENERGY COUPLING IN METHANOGENS

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INTRODUCTION

With the sole exception of the "acetate organisms"[1] and a thermophilic sarcina[2], all methanogens so far isolated have the ability to reduce CO_2 to methane using 4 pairs of electrons derived from H_2, according to equation (1):

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad .....(1) \quad \Delta G^{O} = -131 kJ\ mol^{-1}$$

Thus, the essence of the problem of "how do methanogens make methane?", which so far has largely centred on the study of certain enzymes and cofactors which might be involved in this process[3-6], may with equal justification be formulated as the question "what are the pathways of electron (and proton) transfer during methanogenesis, and how are they coupled to ATP synthesis?". It is the purpose of the present paper to review the currently available answers to this question, laying a greater emphasis than has been usual upon membrane-associated events. We begin with a thermodynamic analysis of bacterial methanogenesis.

THERMODYNAMICS OF METHANOGENESIS

Of the various nutritional modes of growth available to methanogens[7] we shall consider only growth on acetate and growth on H_2/CO_2. During growth on acetate this compound is cleaved[2,8-10] by an unknown mechanism to give CO_2 and CH_4. As stressed by others[5], this dismutation is not an intermolecular electron transfer. The modified standard free energy change for the reaction

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad .....(2)$$

has been given as $-28 kJ.mol^{-1}$[9] or $-31\ kJ.\ mol^{-1}$[3,5], though <u>in vivo</u> the mass-action ratio of this reaction is probably such as to increase its exergony. Thus it is perhaps premature to completely exclude substrate-level phosphorylation as a possible means of generating ATP from this reaction. However, a number of speculative schemes of protonmotive activity directly coupled to reaction (2) have been presented[5,9,10].

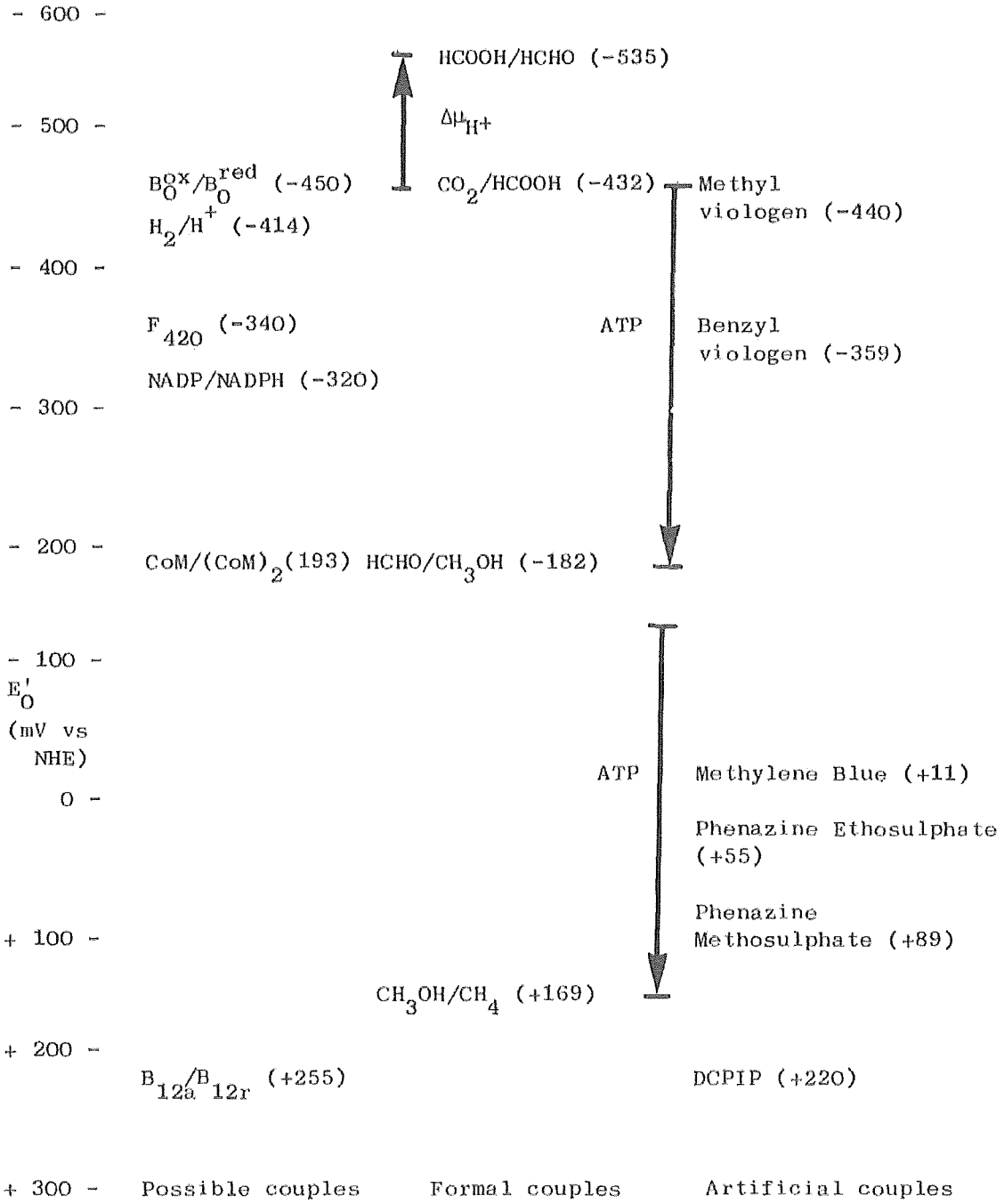

Fig. 1. Redox positions of some reactions of relevance to methanogenesis. Formal couples are given in the central column, possible couples *in vivo* in the left hand column and artificial couples in the right hand column. The redox sign span necessary to make 1 ATP at an *in vivo* phosphorylation potential of -48kJ mol^{-1} is given by the downward pointing arrows, and the necessary energy coupling to drive methanogenesis by the upward pointing arrow.

In view of the membrane permeability of acetate and CO_2, and of their possible uncoupling effects[11,12], we would at present view such schemes with some scepticism, and move on to the autotrophic mode of growth, in which genuine electron transport, coupled to ADP phosphorylation, does undoubtedly take place. As equation (1) indicates, 4 pairs of electrons are required to reduce 1 molecule of CO_2 to methane; the formal energetics of the redox couples at each level of oxidation are very different, with reduction to the level of HCHO by H_2-derived electrons being unfavourable, whilst further reduction to the level of CH_4 is very favourable (Fig. 1). Thus a mechanism for the coupling of the primary and terminal steps of CO_2 reduction is a thermodynamic necessity. Such a coupling, in which the terminal reactions of methanogenesis promote the primary ones, has been observed *in vitro*, and has been termed "the RPG effect", after R.P. Gunsalus who discovered it[5,13]. The nature of this coupling remains obscure, but one possibility, which we should be inclined to favour, is that it is a proton motivated reversed electron transfer of the type displayed by pyridine nucleotide transhydrogenase of mitochondria and of *E. coli*[14]. We discuss this point in some detail later.

In Fig. 1 we have drawn up an energy-level diagram of all known redox couples that may be involved in the transfer of electrons to CO_2. Especially for the formal 1-carbon couples such a tabulation may be only a guide for, apart from methyl-S-CoM[5,6], the precise nature of the redox couples participating in CO_2 reduction remains unknown. However, the positions relative to H_2 of the 1-carbon couples are unlikely to be inaccurate. It is particularly regrettable that, apart from hydrogenase, no membranous redox carrier has unequivocally been identified. In current studies of what is conceded to be a process of electron transport phosphorylation the present emphasis on soluble factors (see later) is truly remarkable. However, we should like to stress that, as Racker[15] has pointed out, *the supernatant of even extracts centrifuged for 2 hours at 105,000 x g contains topologically closed membranous material*. The significance of this in energy coupling, particularly in view of the chemiosmotic theory[3,16], and of derivations thereof (e.g.[17]), will be elaborated more explicitly hereafter. We now turn to a more detailed discussion of the location and nature of the redox compounds presently identified in methanogenic bacteria. Since *Methanobacterium thermoautotrophicum*[18] is undoubtedly the best understood methanogen, we shall confine our attention almost exclusively to this organism, although we realise that some generalisations may well be inapplicable to other organisms, such as the nutritionally more versatile *Methanosarcina barkeri*.

LOCATION AND NATURE OF ELECTRON CARRIERS IN METHANOGENIC BACTERIA

The first notable feature of methanogens is that they apparently contain neither quinones nor cytochromes[3], although spectroscopic evidence for the presence of a *b*-type cytochrome in *Methanosarcina barkeri* has recently been presented[19]. Of the known electron carriers in methanogens, until recently[20] only the hydrogenase, factor F_{420} and coenzyme M derivatives had been reasonably well characterised. The hydrogenase is undoubtedly membrane-bound *in vivo*, though much is displaced during cell breakage: by using a stain for hydrogenase and ATPase, Doddema *et al*[21] were able to demonstrate that the apparatus for electron transport phosphorylation exists on an intracellular membrane

system of M. thermoautotrophicum first described by Zeikus and Wolfe[22] and also present in other methanogens[4,23,24]. Thus whilst most workers who have studied hydrogenase-dependent reactions in these organisms have used "soluble" systems, the ability to identify the location of hydrogenase in intact cells by cytochemical techniques has provided powerful evidence that this enzyme is membrane-bound in vivo (and c.f. also[25]).

F_{420} was the first novel chromophoric compound isolated from methanogens[26] for which a structure has been definitely established[27,28]. It is a deazaflavin and is apparently a 2-electron, 1-proton carrier at neutral pH[29,30]. This, and its polarity, would tend to exclude its participation in the type of redox loop previously proposed[5]. It has a mid-point potential of -340mV (NHE) (Pol, A., unpublished), and can catalyse the transfer of electrons from H_2 to NADP (c.f. Fig. 1). Although no other function for F_{420} has been demonstrated in methanogens (and it is not exclusive to methanogens, being also found in Streptomyces griseus[31]), the finding that it is soluble in the oxidised form yet bound in the reduced form[3,26,32], would seem to exclude its involvement as a direct electron carrying intermediate on the pathway of methanogenesis, as well as lowering its effective in vivo E_h. Walsh[30] has suggested that it participates directly in hydride transfer to NADP[32]. Although F_{420} can participate in the generation of H_2 from formate via a formate hydrogen lyase[33,34], labelling experiments suggest that free formate is neither an intermediate in CO_2 reduction to biomass nor to CH_4 (see e.g.[35]), and thus the first step of CO_2 reduction to methane is not a reversal of the formate hydrogen lyase reaction, as may occur, for instance, in certain Clostridia[36]. It could well be that the role of F_{420} is to reduce NADP for biosynthetic purposes. Our suggestion that this redox carrier is not in fact directly involved in methanogenesis is not inconsistent with the finding that the pathways for CO_2 reduction to biomass and to CH_4 are probably different since they exhibit very different isotope effects[37].

Coenzyme M, 2-mercaptoethane sulphonate, a coenzyme apparently unique to methanogens[38], is thought to be involved in the terminal reaction of methanogenesis[5,6]. The $E_{m,7}$ of the HSCoM/(S-CoM)$_2$ couple is -193 mV (vs NHE)[39]. However, it is the methylated form of the coenzyme which is reduced by H_2-derived electrons to form methane (see e.g.[40]), according to equation (3):

$$CH_3 - S - CoM + H_2 \rightarrow CH_4 + HS - CoM \quad .....(3)$$

This reaction requires a hydrogenase, another protein called "C" and a heat-stable, dialysable cofactor of unknown structure, this ensemble being known as the methyl-CoM reductase complex[40]. In addition, Mg-ATP is required, apparently in catalytic amounts, a point discussed in more detail in a later section. Blaylock and Stadtman[41,42] suggested that vitamin B_{12} might be a methyl carrier, at least in Ms. barkeri, and Wolfe[6] has mentioned a low molecular weight factor, the CDR factor, which is required for CO_2 reduction. Reports exist on chromophoric factors F_{342}[43], F_{430}[43-45] and YFC[46], and Balch and Wolfe[47] mentioned AC-I and AC-II, probably CoM derivatives (c.f[46]), all of which may be electron carriers. It has also been suggested that a succinate/fumarate cycle may be operative in methanogens, although it would appear that fumarate plays an assimilatory role in M. thermoautotrophicum[49]. Very recently, Lancaster[84] has reported on several very interesting membrane-bound and soluble epr-detectable centres in M. bryantii. Finally, Keltjens and Vogels[20] have reviewed their studies of a number of chromophoric factors, all of which are apparently involved in methanogenesis,

and of which at least one, factor B_0 has been shown to be electroactive (Fig. 1). We now turn to a consideration of how electron transport may be coupled to ATP synthesis in methanogens.

PROTONMOTIVATED ATP SYNTHESIS IN METHANOGENIC BACTERIA

In view of the predominance of the chemiosmotic view of energy transduction in bacterial bioenergetics, it seemed logical to assume that a protonmotivated type of ATP synthesis might be occurring in methanogens, particularly since it was known that protonophorus uncouplers decreased the ATP pool in M. bryantii (MoH)[50], and in Methanobacterium strain AZ[51], concomitantly inhibiting methanogenesis. However, more recent experiments undertaken to test this hypothesis yielded the surprising result[52] that ATP synthesis in intact cells of M. thermoautotrophicum elicited by an artificially-imposed pH gradient was insensitive to uncouplers. (Similar experiments in Ms. barkeri[53], in which the external pH was lowered to 2.5, gave uncoupler-sensitive ATP synthesis). It was therefore suggested that the ATP synthesizing apparatus might be located in the internal membrane system in vivo, and, as noted above, staining procedures confirmed this prediction[21]. It should be stressed that centrifugation for 100,000 x g for 1 hour gave a rather low ATPase activity in the pellet, and 3 hours centrifugation at 140,000 x g was necessary to obtain good ATPase (ATP hydrolase) activity in the pellet fraction[52]. Therefore, to those who believe that methanogenic enzymes, by virtue of their association with electron transport carriers linked to ATP synthesis, must be mambrane-associated enzymes, these observations are perfectly consistent with the report of Gunsalus and Wolfe[54] that the terminal enzymes of methanogenesis are in the 100,000 x g supernatant of cell extracts of M. thermautotrophicum prepared in a similar way to those of Doddema et al[21,52]. However, Sauer[24] et al showed that the methane-synthesising apparatus of M. ruminantium was to be found in the pellet of a cell-free extract subjected to 100,000 x g for 30 minutes. In addition, they found that with this preparation methanogenesis from CO_2 was independent of the presence of ATP, although an energised state of the membrane was required, in that methanogenesis was inhibited by protonophorous uncouplers, membrane-permeable phosphonium salts, and lytic concentrations of deoxycholate, and was stimulated by dicyclohexylcarbodiimide, which is believed[76] to block $H^{\pm}$ conducting pores in the F_0 part of $H^{\pm}$ ATPases. Finally, methanogenesis from CH_3-B_{12} is catalysed by the membrane fraction of M. kuzneceovii[55].

Thus we may conclude that there is no evidence so far reported which is inconsistent with the idea that electron transport-linked ATP synthesis, in which electron transfer is ultimately to CO_2, is a classical membrane-associated process, and that methanogenesis from CO_2 is also mainly membrane-associated in vivo.

PROTONMOTIVATED ATP SYNTHESIS IN M. thermoautotrophicum

Membrane vesicles prepared by osmotic lysis are well known to be the most tightly coupled in bacterial electron transport phosphorylation[50]. Thus Doddema et al[21] screened a variety of hydrolytic enzymes in an attempt to prepare protoplasts from this organism, sadly without success; however, preparation of cell-free extracts by French pressure cell

treatment was found to produce a membranous system competent in uncoupler- and ionophore-sensitive ATP synthesis[21]. In this work no terminal electron acceptor was added, and hydrogen-dependent ATP synthesis declined as ATP hydrolysis overwhelmed it in 1-2 minutes[57]. However, phenazine ethosulphate[58] or factor B_0[20] could be added as terminal electron acceptor with concomitant stimulation of ATP synthesis[57]. One remarkable feature of ATP synthesis in this system was that it was sensitive to appropriate concentrations of 3 different inhibitors of the mitochondrial adenine nucleotide translocase enzyme[21] (review[59]), suggesting that such a carrier might be present in these membranes. More recent work[60] has confirmed this suggestion beyond reasonable doubt, since atractylate-sensitive ADP uptake, ATP synthesis and ATP hydrolysis could all be demonstrated. Further, a single polypeptide could be observed to bind radioactive atractyloside during polyacrylamide gel electrophoresis in the presence of triton X-100. Finally, measurement by a flow dialysis technique similar to that described previously[60] of the orientation of the protonmotive force in this system using the membrane-permeable ion distribution method[61] suggested that predominantly 'right-side-out' vesicles, in which the direction of electron-transport-driven H^+ translocation was outwards, contributed to the energised state in this system, (Fig. 2) and that thus ATP synthesis necessitated the participation of an adenine nucleotide translocase enzyme. No ($\leqslant$ 30mV) protonmotivated pH gradient could be detected[57]. It would seem that the key role played by an adenine nucleotide translocase and by the internalised membrane system in M. thermoautotrophicum which necessitates its action, should be taken into account in any appraisal of the phylogeny of methanogens[62-66].

Flow dialysis was performed as described [60] in a medium containing 0.12M sodium bicine pH 7.4, 10mM phenazine ethosulphate and 5 mg vesicle protein [21]. The reaction was started by adding 0.4 µCi of [^{14}C]-dibenzyldimethylammonium bromide (▲ - ▲) (plus 5 µM sodium tetraphenyl borate) or KS^{14}CN (△ - △) each to a final concentration of 7 µM. At the arrow, FCCP was added to a final concentration of 10 µM. T = 60°C. The flow buffer contained 0.0001% w/v resazurin plus 0.01% w/v cystein hydrochloride - tris pH 7.

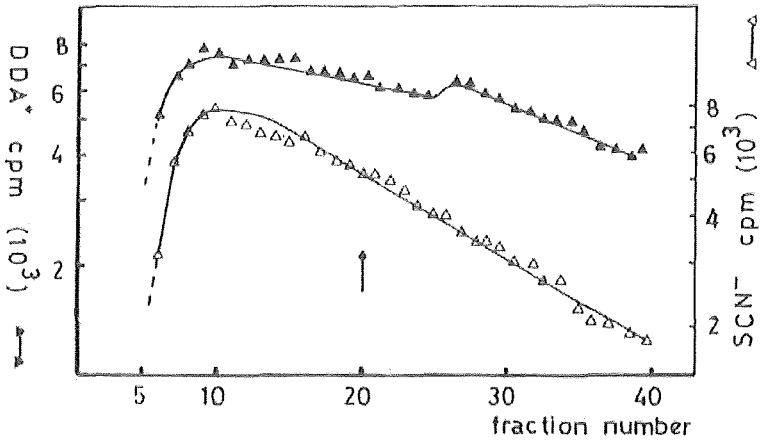

Fig. 2. Orientation of the membrane potential generated as a result of H_2 oxidation by membrane vesicles of M. thermoautotrophicum.

COUPLING OF ELECTRON TRANSPORT, CO_2 REDUCTION AND ATP SYNTHESIS

We have alluded earlier to the tendency of workers in the field of methanogen biochemistry to concentrate upon the "soluble" fraction of cell extracts. We have also suggested that it might be particularly rewarding to reappraise available evidence as supporting the concept that methanogenesis from CO_2 is actually a membrane-associated process in vivo, as is electron transport phosphorylation in other anaerobically-respiring organisms[3,16]. Certainly under autotrophic growth conditions cell growth is entirely dependent upon electron transfer between H_2 and CO_2 and its coupled ATP synthesis. In other words the same electron transfer reactions which are coupled to ATP synthesis are those which are involved in methanogenesis. Further, as would be expected by analogy with other microbial systems[16], such ATP synthesis driven by H_2 oxidation is catalysed by the membrane fraction of cell-free extracts, at least in M. thermoautotrophicum[21] upon which we shall concentrate our analysis.

As mentioned in the introduction, the terminal reactions of electron transfer during methanogenesis must in some way be coupled to the primary endergonic steps of CO_2 reduction. Such a coupling may in principle be either via a 'high-energy' cofactor (such as ATP) or via an energised membrane state[16]. We shall review evidence consistent with the view that the latter theory is correct.

The classical method for testing the involvement of a protonmotivated energised membrane state in any biological process is to see whether that process is sensitive to protonophorous uncouplers of electron transport phosphorylation. It was established for intact cells of M. bryantii (MoH)[50] and strain AZ[51] that protonophorous uncouplers do indeed inhibit methanogenesis though, as the former workers pointed out, 2, 4-dinitrophenol is reduced by methanogens, and thus may not be acting as a pure uncoupler. Sauer et al[24] have also shown that methanogenesis from CO_2 by membrane vesicles of M. ruminantium is sensitive to uncouplers; this is indeed to be expected for the thermodynamic reasons outlined earlier. However, it is not known whether the terminal reaction of methanogenesis, the reduction of methyl-S-CoM by H_2 to CH_4 and HS-CoM, is sensitive to appropriate concentrations of a good uncoupler, although Gunsalus and Wolfe[54] showed that it was inhibited by 2,4-dinitrophenol. Since both the HS-CoM$(S\text{-CoM})_2$ and CH_3OH/CH_4 couples lie on the oxidising side of H_2, one can say with certainty that reaction (3) is exergonic, although the exact thermodynamics cannot be calculated from available information. Thus, an experimental determination of the equilibrium constant for the reaction of equation (3) will be eagerly awaited. It should by mentioned that reaction (4):

$$CH_3\text{-S-CoM} + \text{HS-CoM} \rightarrow CH_4 + (\text{S-CoM})_2 \quad .....(4)$$

followed by $(S\text{-CoM})_2$ reduction does not yet appear to have been excluded as an intermediary pathway in reaction systems designed to assay the reaction of equation (3). It is thus remarkable that the exergonic reaction (3) has, in the hands of most workers, an absolute requirement for ATP, although this role is "catalytic", 1 molecule of ATP being required per fifteen[54] or five[67] CH_4 molecules formed. ADP (which may form ATP via the adenylate kinase reaction in this organism) sustained 50% of the rate achieved with ATP, but non-hydrolysable analogues of ATP were ineffective[54] at promoting the reaction. Recently, Shapiro and Wolfe[68] also found a "catalytic" requirement for ATP in the methylation

of CoM by methanol in extracts of M. barkeri, 85 methyl groups being transferred per molecule consumed. Earlier, in an extract of M. bryantii (MoH), Roberton and Wolfe[69] had shown that resynthesis of ATP during methanogenesis from CO_2, although taking place, was not able to account for the puzzling lack of stoichiometry, and also that, once ATP had reacted, its presence was unnecessary for ongoing methanogenesis. There are a number of possible explanations for the role of ATP in this reaction which suggest themselves to the bioenergeticist: (i) it may act to block a proton leak, as found for ATP in chloroplasts[70] and for GTP in brown fat mitochondria[71], (ii) it may act to create an energised membrane state by being hydrolysed but subsequently and slowly resynthesised; such an energised state might be only a kinetic and not a thermodynamic requirement, (iii) it may act to adenylate or phosphorylate an enzyme or cofactor. Attempts by Gunsalus and Wolfe[54] to isolate such a covalently-modified form of the enzyme complex, were unsuccessful. In view of the fact that the ATP requirement disappears once ATP has reacted[69], we are inclined presently to favour this last possibility.

INHIBITORS OF METHANE GENERATION AND H^+-ATPase

It would be useful to have at our disposal a family of electron transport and ATPase inhibitors, with which to dissect out the various components in the train of events which comprise the bioenergetic pathway in methanogens. Regrettably, those currently available are few in number, and (in the case of possible electron transport inhibitors) their mode or site of action is unknown. On a molar basis, amongst the most potent inhibitors of electron transport are the viologen dyes[72], with E_0' values as shown in Fig. 1. They may certainly be preferentially reduced by the hydrogenase in cell-free extracts[40]. Nitrous oxide blocks methanogenesis from CO_2[73] but not from CH_3-S-CoM. Nitrite and 1,10-o-phenanthroline are capable of inhibiting the reduction of CH_3-S-CoM. Bromoethane suphonate is a potent inhibitor of methanogenesis from CH_3-S-CoM[74]; whether it inhibits earlier stages of CO_2 reduction, or methanogenesis from methanol in M. barkeri[68] is apparently unknown. Halogenated methane analogues are also potent inhibitors of methanogenesis[75], although the inhibition by long-chain fatty acids[75] may be ascribed to an uncoupling action.

Of known ATPase inhibitors[76] efrapeptin and dicyclohexyl carbodiimide (DCCD) are active against the membrane-bound ATPase of M. thermoautotrophicum but oligomycin, Dio-9, venturicidin and tributyltin are not[57]. Note that assays of the effectiveness of polypeptide antibiotics at 65°C should be performed with caution. Since extracts of M. thermoautotrophicum usually contain a significant adenylate kinase activity, it is useful to note that diadenosine pentaphosphate (Ap_5A)[77] is a potent inhibitor of this reaction in this organism[57] (R.K. Thauer, pers. comm.).

Other energy-linked reactions of methanogens

Essentially nothing is known of putatively protonmotivated solute uptake in methanogens, although staining procedures in M. thermoautotrophicum show some ATPase activity in the cytoplasmic membrane[21]. However, Balch and Wolfe[47] did demonstrate an energy-dependent uptake of CoM and methyl CoM in M. ruminantium. Apart from the work on adenine nucleotide translocase[60] no studies have yet appeared of other active transport systems in methanogens.

Growth yields and the P/2e$^-$ ratio in methanogens

Thauer et al[3] suggested from a consideration of the poor growth yields then found, and from the thermodynamics of autotrophic growth[78] that methanogens should have a P/2e$^-$ ratio of 1. We are inclined to think this suggestion a little premature for a number of reasons: (i) recent thinking[79-81] suggests that there is no need for P/2e$^-$ ratios to have integral values, (ii) increases in growth yield have been achieved through improvements in the growth medium, particularly the addition of nickel[44-82], (iii) the subtlety of the coupling between the various electron transfer reactions of methanogenesis, and our ignorance of the actual carriers involved, indicates that one should not truly speak of a single P/2e$^-$ ratio being displayed by these organisms. We may also note that the efficiency of growth is a function of H_2 and CO_2 concentration[12]. Thus as yet no approach to the estimation of the P/2e$^-$ ratio seems compelling. In this regard it is regrettable that no attempts to measure the $\rightarrow H^+/2e^-$ ratio using an H_2-pulse method have yet been described, although, since the presence of carbonic anhydrase is required for this assay[83], CO_2 cannot be used as a terminal electron acceptor[57].

SUMMARY AND PROSPECT

The renaissance of interest in the methanogenic bacteria which followed improvements in the techniques for growing these strict anaerobes was fostered by a more widespread realisation of their importance in systems for energy transduction using renewable resources. It is somewhat paradoxical therefore that so little is still known of the bioenergetics of these organisms themselves. It seems too early to speculate about energy-linked reactions in methanol- or acetate- grown cells, but from presently available data the following concept emerges to describe energy coupling in autotrophically-grown cells.

The reduction step from the formate to formaldehyde levels of reduction is thermodynamically unfavourable, and requires an input of energy, which is supplied by an energised membrane state. The necessary reversed electron transfer makes the reduction of CO_2 to CH_4 uncoupler-sensitive. In this respect it is important to realize that all soluble systems so far described contain membrane vesicles. Further reduction of the C_1 unit from the level of formaldehyde to that of methyl-S-CoM follows a presently unknown pathway. The final reduction step from the methyl-S-CoM level to CH_4 requires catalytic amounts of ATP, probably to adenylate or phosphorylate one of the components of the methylreductase complex. Uncoupler sensitivity at this stage seems unlikely *in vivo*. One may speculate that, at least in *M. thermoautotrophicum*, CO_2 reduction to CH_4 takes place within a specialised "methanochondrion" organelle.

Purification and reconstitution of the enzymes and soluble cofactors involved in methanogenesis will obviously be needed to study further the electron transfer pathways of methanogens, and identification of the undoubtedly numerous membrane-bound electron carriers may be anticipated together with study of their redox potentials and cellular location. The sensitivity of methanogenesis to ionophores and phospholipases remains, importantly, to be established. The isolation of sphaeroplasts of methanogenic bacteria would prove most useful for furthering our understanding of energy coupling in these unique and existing bacteria.

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Note added in proof; Kell et al.

Very recently Sauer and coworkers[85,86] have demonstrated that methanogenesis from CO_2 is catalysed by the membrane fraction of M. thermoautotrophicum in the absence of added ATP, is sensitive to the ionophore valinomycin and possesses certain other properties consistent with the view advanced here that the energy-coupling system in this organism is located in vivo on an intracellular membrane system.