JOURNAL OF BACTERIOLOGY, Apr. 1970, p. 43-51

Vol. 102, No. 1
Printed in U.S.A.

Adenosine Triphosphate Pools in *Methanobacterium*

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Received for publication 6 November 1969

Certain aspects of adenosine triphosphate (ATP) metabolism in the strict anaerobe *Methanobacterium* strain M.o.H. have been investigated. Results of growth yield studies suggest that ATP conservation is very inefficient (0.06 mole of ATP per mole of hydrogen) under the conditions used to grow the bacterium in a fermentor. Experiments designed to demonstrate net ATP formation in cell-free extracts were negative. In whole-cell studies, substances which decreased ATP pool levels and increased adenosine monophosphate (AMP) pool levels were air, chloroform, 2,4-dinitrophenol, carbonylcyanide-*m*-chlorophenylhydrazone, and pentachlorophenol. The results suggest that the latter compounds act either as inhibitors of electron transport or as uncouplers of an energy-linked process. All the above compounds also inhibit methane formation in cell-free extracts, an ATP-requiring process. Methods are described for estimation of ATP, adenosine diphosphate (ADP), and AMP in whole cells, with a sensitivity in the range of 10 to 200 pmoles. An apparatus for quick sampling from an anaerobic suspension of whole cells also is described.

Although most work on oxidative phosphorylation in bacteria has been done with aerobic systems, there have been several demonstrations of oxidative phosphorylation, under anaerobic conditions, by cell-free extracts of facultative anaerobes with nitrate as the terminal electron acceptor. The bacteria on which such studies have been made include *Escherichia coli* (10), *Pseudomonas denitrificans* (9), *P. aeruginosa* (19), *Nitrobacter winogradskyi* (6), and *Micrococcus dentrificans* (15).

More recently, Peck has demonstrated oxidative phosphorylation in extracts of the strict anaerobe, *Desulfovibrio gigas* (11, 12). Here, P/2e ratios of 0.12 were obtained with hydrogen as the electron donor and sulfite as electron acceptor.

Another bacterium which may obtain its energy by anaerobic oxidative phosphorylation is *Methanobacterium* strain M.o.H., since it is not obvious how its substrates, hydrogen and carbon dioxide, can provide energy by substrate level phosphorylation. In the present study of this bacterium, several different compounds, known to modify energy metabolism or electron transport, have been examined for effects on methane production and on adenine nucleotide pools in whole cells, as well as on methane production in cell extracts.

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MATERIALS AND METHODS

Culture methods. *Methanobacterium* strain M.o.H was mass-cultured in 12-liter amounts as previously described (3).

Dry-weight measurements. Two preweighed filter membranes (mean pore size, 0.45 nm; Millipore Corp., Bedford, Mass.) were placed one on top of the other in a vacuum suction apparatus. Cells from a 5- to 20-ml sample of cell suspension were retained on the filter and were washed with about 2 ml of water. The filters were separated and dried at 110 C to constant weight. The bottom filter served as a control.

Gas measurements. The apparatus used to determine the uptake of hydrogen and carbon dioxide and the production of methane during growth in a 12-liter fermentor are shown in Fig. 1. A mixture of hydrogen and carbon dioxide (80:20) from the cylinder (A) was passed through a 100-ml, soap bubble, gas flow meter (C) at a rate of 10 to 50 liters per hr, depending on the stage of growth. The pressure of the gas in (C) was calculated from the atmospheric pressure and from the reading obtained on a manometer (B) filled with Brodie solution. Any soap was removed from the gas by passing it through the water vessel (D). All the taps labeled E were bleeds, through which water could be removed from the lines when necessary. Vessel (F) was a serum bottle, stoppered with a serum cap, from which gas samples could be removed for analysis. The gas was passed over heated copper (G) to remove any traces of oxygen, and then through a sterile glass wool plug (H) into the fermentor (I). Gases from the exit port (J) of the fermentor were passed through a tube of $MgClO_4$ (L) to remove water, though, during the intervals between readings,

this was short-circuited by appropriate opening and shutting of the taps (K). The pressure in the exit line was determined from the reading of the manometer (M) and the atmospheric pressure. Exit gas samples were removed for analysis through the serum cap of bottle (N), and the rate of exit gas flow was determined with the soap bubble meter (O).

The constitution of gas samples, removed from the entrance (F) or the exit (N), was analyzed as follows. Methane was measured in duplicate in 0.4-ml samples by using a Packard model 7500 series gas chromatograph which contained a silica gel column connected to a hydrogen flame detector. Carbon dioxide was measured in duplicate in 1-ml samples with a Beckman GC-2 gas chromatograph which contained a silica gel column and a thermal conductivity detector. Total gas flow from the fermentor was determined from measurement of gas flow rates (meters C and O), and pressure corrections were applied. Since the quantity of total gases flowing in and out and the fractions of carbon dioxide and methane in these samples were known, the hydrogen content of the influent and effluent gases could be obtained by subtraction. Finally, the uptake of carbon dioxide and hydrogen and the production of methane were calculated by comparing the quantity of each gas which was entering and leaving the fermentor.

Spectrophotometric assays of ATP, ADP, and AMP standards. Adenine nucleotide standards (mM) were analyzed by spectrophotometric assay, before making a 100-fold dilution for use in the firefly luciferin-luciferase assay. ATP was standardized by use of the Boehringer ATP-kit (Calbiochem.), which measures reduced nicotinamide adenine dinucleotide (NADH) oxidation coupled to 3-phosphoglycerate phosphorylation and reduction via 3-phosphoglycerate-kinase and glyceraldehyde-3-phosphate dehydrogenase. ADP was standardized by use of the coupled pyruvate kinase and lactate dehydrogenase assay (1) which measures NADH oxidation. AMP was standardized by use of 5'-adenylic acid deaminase (14). An extinction coefficient of 8,080 per cm per mole for the change in extinction at 265 nm was used (8).

Measurement of ATP, ADP, and AMP levels in whole cells. Cells (250 to 500 ml) were removed from a 12-liter fermentor and centrifuged at 8,000 × *g* at room temperature for 15 min in a Sorvall RC-2 centrifuge. The cells were suspended in 50 mM potassium TES [N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid] buffer (*p*H 7.4) which previously had been degassed under vacuum and equilibrated with hydrogen. The bacterial dry weight was 1 to 4 mg per ml; 40 ml of suspension was placed in a rapid sampling apparatus (Fig. 2) based on the design of Knight (7).

A hydrogen and carbon dioxide mixture (80:20) was bubbled through the suspension at a rate of approximately 25 ml per min. Reactions were carried out at room temperature. The cell suspension was rapidly stirred by a water-driven magnetic stirrer. A soap bubble gas flow meter was attached to the gas exit, and the gas phase was kept at a positive pressure by bubbling the effluent gas into a cylinder of water, with the outlet 20 cm below the surface. Gas samples could be removed from above the surface of the cell suspension, or additions could be made to the cell suspension, by inserting a syringe through the serum cap. Samples of cell suspension were obtained by opening the bottom tap and allowing 0.8 to 1.5 ml of the suspension run into 1.0 ml of ice-cold 30% (w/v) perchloric acid. From this particular bacterium, it was difficult to extract all the adenine nucleotide, but it was empirically found that, by leaving the suspension for 3 hr at 0 C, a maximal quantity was extracted. The suspension was then neutralized with 4 N KOH; the

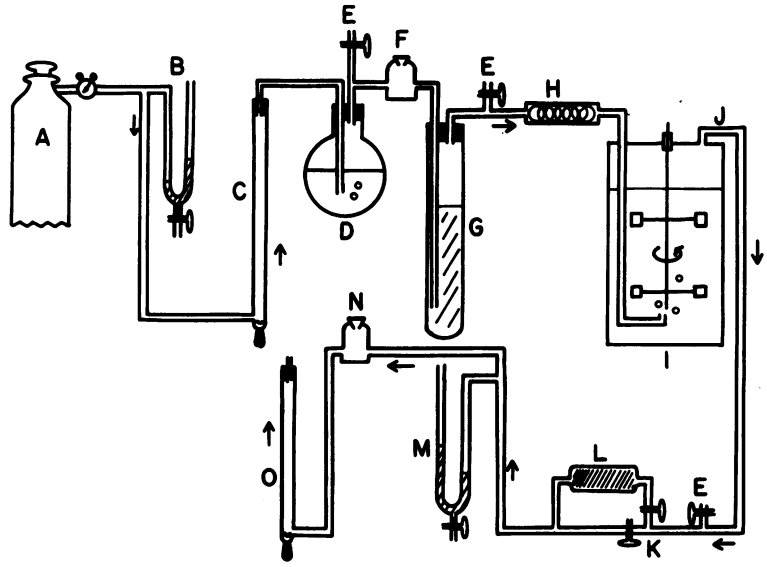

FIG. 1. *Apparatus for measuring gas uptake and evolution during growth of the organism in 11 liters of medium.*

precipitate was removed by centrifugation, and the supernatant fraction was removed with a Pasteur pipette.

ATP was assayed by the firefly luciferin-luciferase assay, essentially as described by Cole, Wimpenny, and Hughes (4). Worthington freeze-dried firefly extract, which contained 20 mM magnesium sulfate and 50 mM potassium arsenate buffer when reconstituted, was suspended in water at a concentration of 10 mg/ml. The suspension was allowed to stand for 3 to 6 hr at 0 C; it was then centrifuged, and the supernatant solution was used for the assay. To an acid-cleaned glass scintillation vial was added 5 ml of a solution which contained 4 mM magnesium sulfate, 16 mM potassium TES buffer (*p*H 7.4), and 10 μliters of the ATP sample; finally 25 μliters of firefly extract was added. Within 7 sec of adding and mixing the firefly enzyme, the vial was introduced into the counting chamber of a Mark I Nuclear-Chicago liquid scintillation computer model 6860, and the light flashes were counted for four successive periods of 6 sec each. The first count of 6 sec was usually high and was discarded; the second and third counts were similar and were averaged. The scintillation counter channel was set as for tritium counting (maximal amplifier gain in the attenuation, upper gate 9.9 v, lower gate 0.5 v), and the photomultiplier coincidence circuit was switched out. Background counts were subtracted, and each ATP sample was measured in duplicate. Between 96 and 100% recovery of ATP was found when ATP standards were subjected to the perchloric acid treatment and neutralization described above.

Table 1 shows a typical standard assay for ATP and the standard assays which were developed to estimate ATP + ADP and ATP + ADP + AMP. A standard curve must be established for each experiment. For the ATP assay, a 1 mM ATP solution was diluted 100-fold, and samples were added to the firefly assay. It will be seen that the assay is linear, and that AMP and ADP do not interfere with the assay.

For the AMP + ADP + ATP assay, a 0.5-ml sample of 10 μM AMP (or the neutralized supernatant solution from extracted cells, or a suitable mixture of standards) was mixed with 0.2 μmoles of tricyclohexylammonium phosphoenolpyruvate, 10 μmoles of magnesium sulfate, 74 μmoles of potassium TES buffer (*p*H 7.4), 50 μg of pyruvate kinase, and 25 μg of myokinase (or 10 μmoles of ATP when used with pure standards); the final volume was 1 ml. The assay mixture was incubated at 37 C for 1 hr. A 10-μliter sample was then removed and assayed for ATP, exactly as described above. The results in Table 1 show that the recovery of AMP or a mixture of adenine nucleotides is good.

The ADP + ATP assay was performed by adding 5 ml of the magnesium sulfate-buffer solution, 0.1 μmoles of tricyclohexylammonium phosphoenolpyruvate, 10 μliters of 10 μM ADP (or a suitable mixture of standards, or the neutralized cell supernatant solution) and 10 μg of pyruvate kinase to the scintillation vial. After incubation for 30 min at room temperature, 25 μliters of firefly enzyme was added, and the reaction mixture was counted as described above. The results in Table 1 show that the assay is linear, but that the counts are 10% lower than expected as judged from the ATP counts. This is because both the phosphoenolpyruvate salt and the pyruvate kinase enzyme separately interfere with the assay. This difficulty may

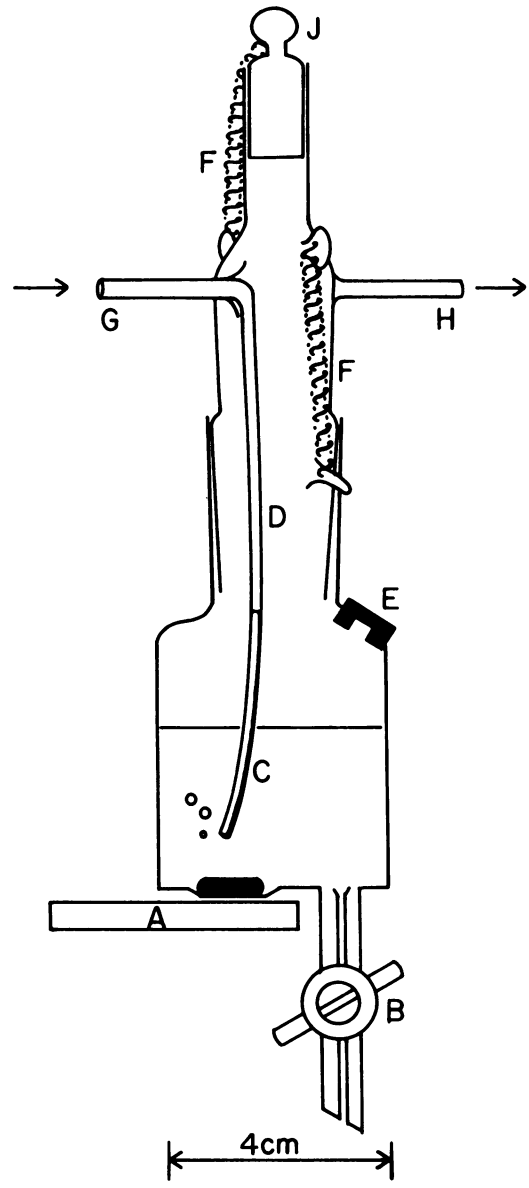

FIG. 2. *Scale drawing of rapid-sampling apparatus (external width, 4.4 cm) used in experiments in which adenine nucleotide pool levels were measured. (A) Water driven magnetic stirrer, (B) tap for rapid sampling, (C) Teflon tubing immersed in cell suspension, (D) glass tube carrying gas mixture to cells, (E) serum cap, (F) spring, (G) gas inlet, (H) gas outlet, (J) ground glass stopper, that was removed to add initial cell suspension.*

TABLE 1. *Assay of adenine nucleotide[a] standards by using the firefly luciferin-luciferase assay*

Assay vial	Additions (pmoles)			Counts per 6 sec[b]	ATP (pmoles)	
	ATP	ADP	AMP		Measured	Expected
For ATP						
1				0	0	0
2	50			8,988	50	50
3	100			18,081	101	100
4	150			26,681	149	150
5	200			35,826	200	200
6	50	100		9,172	51	50
7	50		100	8,860	49	50
8	100	100		18,306	102	100
9	100		100	17,502	98	100
10		100		0	0	0
For ATP + ADP						
1				0	0	0
2[c]	100			16,687	100	100
3[c]	200			33,452	200	200
4		50		7,429	45	50
5		100		14,779	89	100
6		200		29,768	179	200
7		50	100	7,980	48	50
8		100	100	14,704	89	100
9	50	50		14,598	88	100
10	50	50	50	14,769	89	100
11	50	50	200	14,731	89	100
For ATP + ADP + AMP						
1				0	0	0
2	100			16,130	100	100
3			50	8,307	52	50
4			100	15,372	96	100
5[d]	25	25	29	13,069	81	79

[a] Adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine monophosphate, AMP.

[b] Background subtracted from values presented.

[c] No phosphoenolpyruvate or pyruvate kinase was added.

[d] Spectrophotometric assay of the ADP showed it contained AMP. Hence equal amounts of adenine nucleotides were not present.

be overcome by use of an ADP standard or by application of a routine standard correction. AMP does not interfere with the assay.

We have occasionally found a phenomenon which may interfere with the ADP + ATP assay. Instead of the successive 6-sec counts being constant, a few firefly enzyme preparations cause the counts to increase with time. (This is not observed in the ATP assay or the AMP + ADP + ATP assay.) This effect may be from the combined action of firefly enzyme myokinase and the added pyruvate kinase on AMP. When it occurred, we used a value for calculations which was obtained by extrapolation of the count rate back to zero time. This phenomenon has been such that only about 10% of the preparations warrant applying the correction, which amounts to between 10 and 30% of the value obtained, depending on the initial concentration of ATP + ADP.

To estimate each adenine nucleotide in the perchloric acid extract of cells, ATP, ADP + ATP, and AMP + ADP + ATP are measured in separate portions of the sample; each is then calculated by appropriate subtraction.

RESULTS

Molar growth yields. Results of a growth study of *Methanobacterium* strain M.o.H. are shown in Fig. 3. Medium (11 liters) at 40 C in the fermentor was inoculated with 200 ml of cells, and gas samples were analyzed at the times shown; 20-ml samples were used for dry-weight estimations. It can be seen that the carbon dioxide utilized matches the methane formed, and that hydrogen utilization was between 3.7 and 3.85 times as

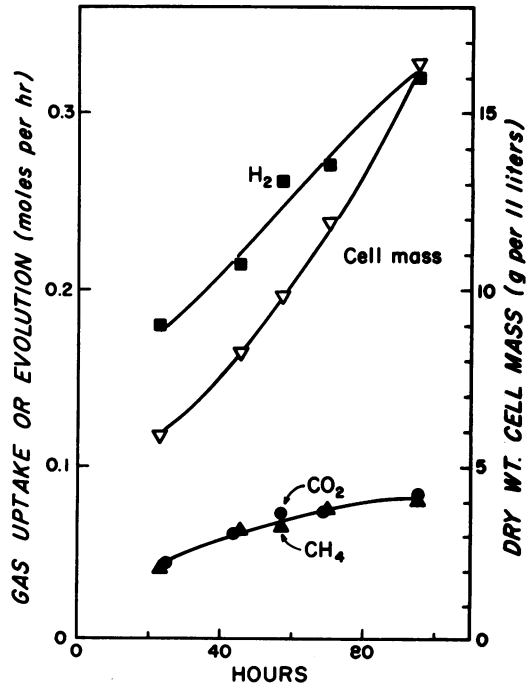

FIG. 3. *Relation of cell mass, uptake of hydrogen and carbon dioxide, and formation of methane during a growth period of 24 to 96 hr. Symbols: ■, hydrogen uptake; ●, carbon dioxide uptake; ▲, methane formation; ▽, cell mass. Liquid volume, 11 liters. Initial cell mass and insoluble components of the medium amounted to 4.7 g per 11 liters of medium.*

great. The theoretical ratio of 4 never has been obtained, indicating that a small amount of reducing power may be obtained from components of the medium. An analysis of the results shows that the maximal growth yield found was 0.62 g (dry weight) per mole of hydrogen. Values between 0.55 and 0.62 were found throughout the time period studied.

Effect of air on methane production and adenine nucleotide levels. Fresh cells harvested from an actively growing culture were suspended in potassium TES buffer (*p*H 7.4) and were gassed with hydrogen and carbon dioxide (Fig. 4). It may be seen from the lag that exposure of cells to air (in the absence of hydrogen) as they were harvested greatly inhibited methane production; after a 3-hr lag, a sudden resumption of methane production occurred. When methane production reached a maximum, air was bled into the hydrogen-carbon dioxide mixture which was flowing through the cell suspension (1.55 ml of air per min, mixed with 30 ml of hydrogen-carbon dioxide per min). It will be seen that there was a slow decline in methane production and that it took 1.5 hr before methane production was 90% inhibited, indicating that cells which are actively metabolizing hydrogen are relatively resistant to inhibition by oxygen.

The ATP levels in the cells increased with commencement of methane production, whereas the AMP levels declined. The ATP levels decreased again as methane production was inhibited by oxygen. ADP levels did not change radically throughout the experiment. It should be noted that the adenine nucleotide recovered was constant throughout the experiment.

Effect of chloroform on methane production and adenine nucleotide levels. Chloroform is a potent inhibitor of methanogenesis (17); it was therefore of interest to examine the effect of chloroform on the ATP levels in whole cells (Fig. 5). As usual, there was a lag in methane production, during which ATP levels were low and AMP levels were high. When both the methane production and the ATP level had reached a maximum, 0.5 mM

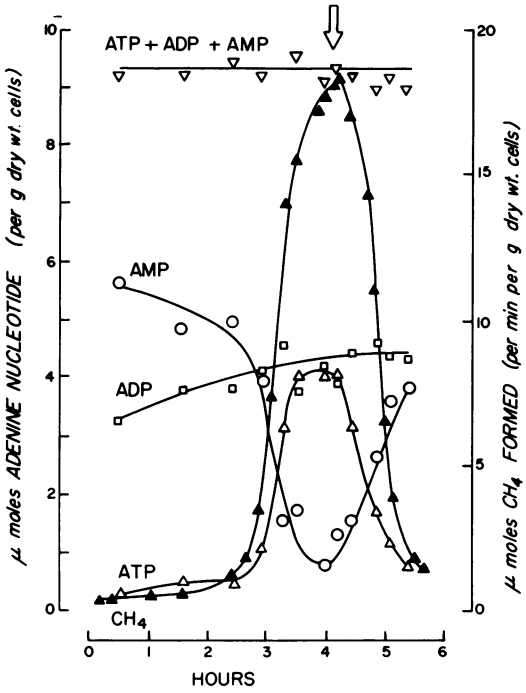

FIG. 4. *Effect of air on adenine nucleotide pools and methane production in whole cells. Initially, 40 ml of cells (2.94 mg dry weight/ml) were present, and a mixture of hydrogen-carbon dioxide (80:20) was bubbled through at a rate of 30 ml/min. At the arrow, air (1.55 ml/min) was bled in with the hydrogen-carbon dioxide mixture. Temperature, 24 C. Symbols: ▲, methane formation; △, ATP; □, ADP; ○, AMP; ▽, ATP + ADP + AMP.*

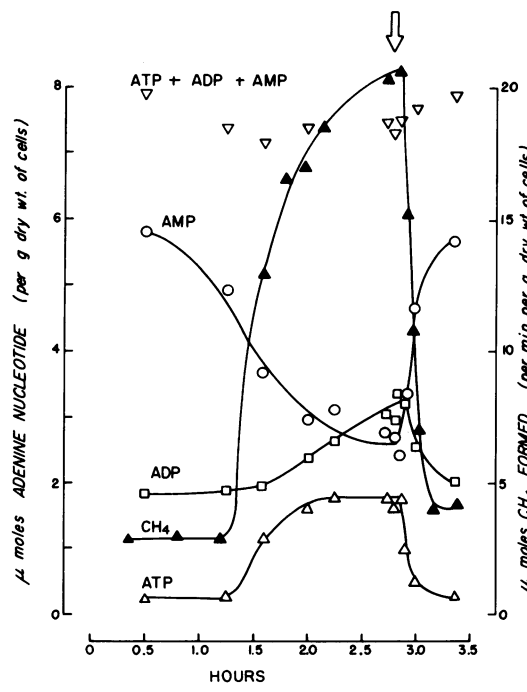

FIG. 5. *Effect of chloroform on adenine nucleotide pools and methane production in whole cells. Initially 40 ml of cells (8.9 mg dry weight/ml) were present, and a mixture of hydrogen-carbon dioxide (80:20) was bubbled through at a rate of 115 ml/min. At the arrow, chloroform (0.5 mM, final concentration) was added. Temperature, 25 C. Symbols: ▲, methane formation; △, ATP; □, ADP; ○, AMP; ▽, ATP + ADP + AMP.*

chloroform was added. Both ATP levels and methane production rapidly fell to low levels.

Effect of uncouplers of oxidative phosphorylation on methane production. Three compounds that uncouple oxidative phosphorylation in mitochondria, 2,4-dinitrophenol (DNP), carbonylcyanide-*m*-chlorophenylhydrazone (CCP), and pentachlorophenol (PCP), were tested for their ability to inhibit methane formation by whole cells. Over 95% inhibition is caused by 10^{-4} M DNP, 4×10^{-5} M CCP, and 5×10^{-4} M PCP (Fig. 6). The inhibition caused by 2×10^{-5} M DNP is reversed in time (in this case, after nearly 3 hr), and methane production rapidly increased. About 40% inhibition is caused by 10^{-4} M PCP, and the inhibition appears to be nearly constant with time. It should be noted that, in this experiment, the uncoupler was added while the cells were in a lag phase of methane production caused by harvesting and air shock.

Experiments in which CCP and PCP were added to whole cells producing methane at maximal rates are shown in Fig. 7 and Fig. 8. In the former, 5×10^{-5} M CCP caused a decrease in both the ATP level and in the methane production. ADP and AMP levels were not measured in this experiment. In the latter, 5×10^{-4} M PCP caused

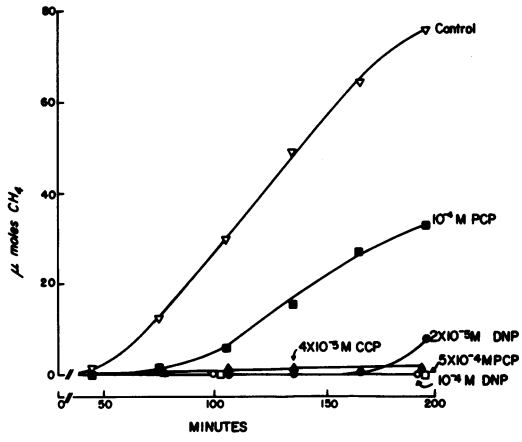

FIG. 6. *Effect of 2,4-dinitrophenol (DNP), carbonyl cyanide-m-chlorophenylhydrazone (CCP), and pentachlorophenol (PCP) on methane production in whole cells. Cells [4.1 mg (dry weight) suspended in 1 ml of 50 mM-potassium TES, pH 7.4] were placed in the main compartment of a Warburg flask, and uncoupler in 0.5 ml of buffer was placed in the side arm. The flasks were gassed with a mixture of hydrogen-carbon dioxide (80:20) for 25 min at room temperature, tipped, and shaken in a water bath at 37 C. Methane formation was measured. Symbols: ▽, control; ●, 2×10^{-5} M DNP; ○, 10^{-4} M DNP; ▲, 4×10^{-5} M CCP; ■, 10^{-4} M PCP; □, 5×10^{-4} M PCP.*

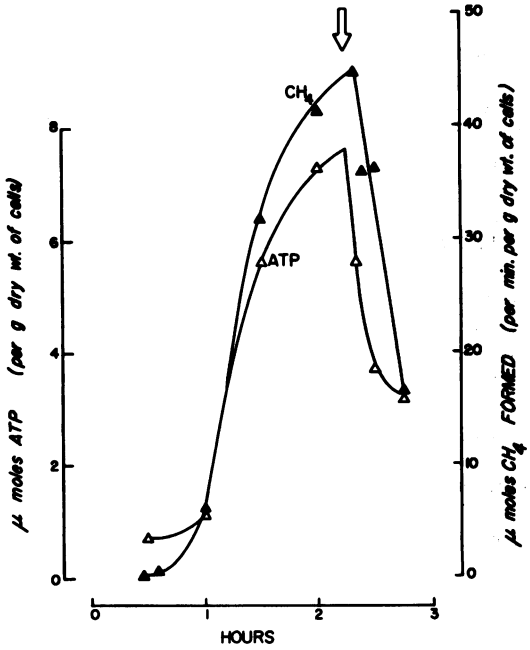

FIG. 7. *Effect of carbonyl cyanide-m-chlorophenylhydrazone (CCP) on methane production and ATP levels in whole cells. Initially 40 ml of cells [8.51 mg (dry weight/ml)] were present, and a mixture of hydrogen-carbon dioxide (80:20) was bubbled through at a rate of 7.9 ml/min. At the arrow, CCP (final concentration, 5×10^{-5} M) dissolved in 38 μliters of dimethylformamide was added. Temperature, 25 C. Symbols: ▲, methane formation; △, ATP.*

very rapid inhibition (75%) of methane production, and the ATP level also dropped 70%.

Several other uncouplers of oxidative phosphorylation in mitochondria have been tested against methane production in whole cells. Tetrachloro-trifluoromethylbenzimidazole (5×10^{-5} M) gave 80% inhibition, dicoumarol (10^{-4} M) gave 40% inhibition, and tribromoimidazole (10^{-3} M) gave 40% inhibition. The rate of permeability of these compounds into the cells is unknown.

Correlation of ATP levels and methane production in whole cells. When the values for methane production and adenine nucleotide levels are plotted against each other (Fig. 4), it may be readily seen that there is a linear relationship between ATP levels and the rate of methane production; there is an inverse relationship between AMP levels and the rate of methane production. The ADP level stays about constant. If follows that there will be a linear relationship between energy charge (ATP concentration plus half ADP concentration, divided by total adenine nucleotide) and methane formation.

Effect of uncouplers on methane production by cell extracts. It was of interest to show whether the inhibitory effect of uncouplers on methane production in whole cells would be observed also in cell extracts. DNP, CCP, and PCP cause inhibition of methane production in cell extracts (Fig. 9–11). The inhibition by DNP (Fig. 9) is reversed with time, probably because of the reduction of the uncoupler, as judged by the change in spectrum of the DNP. CCP at 2×10^{-4} M (Fig. 10) caused about 65% inhibition at 30 min, and PCP at 10^{-3} M caused about 75% inhibition at 45 min (Fig. 11); these inhibitions are not reversed by time, suggesting that the uncouplers are not reduced.

DISCUSSION

It is not known whether the strict anaerobe *Methanobacterium* strain M.o.H. obtains its energy by oxidative phosphorylation or by substrate level phosphorylation. The bacterium catalyzes the overall reaction $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$, but, as yet, no substrate level phosphorylation involving C_1 compounds has been demonstrated in cell extracts.

As a first approach to the problem of how this bacterium conserves its energy, we examined the growth yield. Stadtman (13) has calculated

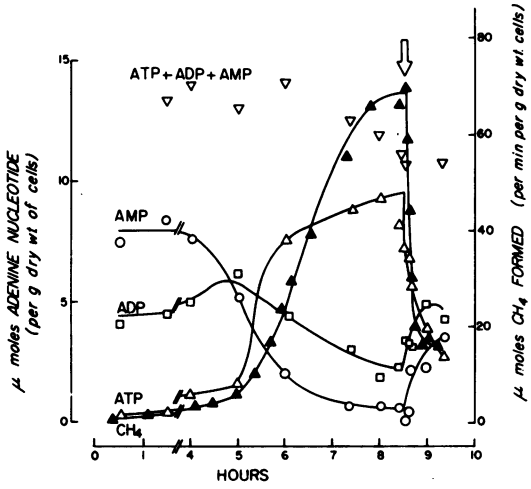

Fig. 8. Effect of pentachlorophenol (PCP) on adenine nucleotide pools and methane production in whole cells. Initially, 40 ml of cells [1.1 mg (dry weight)/ml] were present, and a mixture of hydrogen-carbon dioxide (80:20) was bubbled through at a rate of 24 ml/min. At the arrow, PCP (5×10^{-4} M, final concentration) was added. Temperature, 23 C. Symbols: ▲, methane; △, ATP; □, ADP; ○, AMP; ▽, ATP + ADP + AMP. The decrease in total nucleotide pool levels after 7 hr is due to incomplete extraction of the cells.

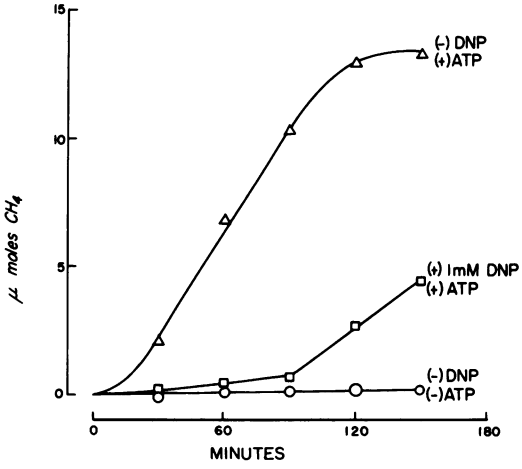

FIG. 9. *Effect of 2,4-dinitrophenol (DNP) on methane production by cell-free extracts. Cell-free extract (25 mg of protein, suspended in 1 ml of 100 mM potassium TES buffer, pH 7.1) was placed in the main compartment of a Warburg flask; 5 μmoles of ATP or DNP, or both, in 0.5 ml of buffer was placed in the side arm where indicated. Flasks were gassed for 20 min at 0 C with hydrogen-carbon dioxide (80:20), tipped, and then shaken at 37 C in a water bath. Symbols: △, no DNP; □, 1 mM DNP; ○, no DNP and no ATP.*

that the free energy of the above reaction is −32 kcal per mole of methane formed. The maximal growth yield, obtained during the growth conditions in the fermentor, was 0.62 g (dry weight) per mole of hydrogen. This is equivalent to 0.06 mole of ATP, assuming the Y_{ATP} equals 10.5 g (dry weight) per mole of ATP (2). If the free energy of ATP hydrolysis to inorganic orthophosphate and ADP is 8 kcal/mole, the energy conservation by the bacterium is about 6% efficient; if there is one phosphorylation per pair of electrons utilized, the reaction is about 94% uncoupled. This represents growth efficiency under the best conditions of mass culture which we have developed so far (3), but we realize that certain unknown factors in the hydrogen-carbon dioxide mass-culture system must be limiting. Stadtman (13) also reported low growth yields for the culture, known as *Methanobacillus omelianskii* [0.78 to 0.94 g (dry weight) per mole of ethyl alcohol, or 0.39 to 0.47 g (dry weight) per pair of electrons per mole of hydrogen transferred to methane and water]. The bacterium used in the present work was the methanogenic strain isolated from the mixed culture, *M. omelianskii*. These results give some indications of the maximal ATP formation one might look for in cell-free extracts. However, when we attempted to measure

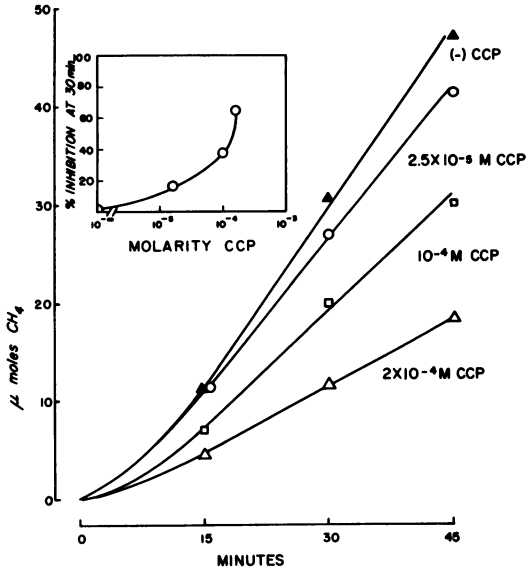

FIG. 10. *Effect of carbonyl cyanide-m-chlorophenyl hydrazone (CCP) on methane production by cell-free extracts. Cell-free extract (45.4 mg of protein suspended in 1 ml of 50 mM potassium TES, pH 7.1) was placed in the main compartment of a Warburg flask, and 10 μmoles of ATP and CCP (added in 5 μliters of dimethylformamide) in 0.5 ml of buffer were placed in the side arm. Flasks were treated as described in the legend to Fig. 9. Symbols: ▲, no CCP; ○, 2.5 × 10⁻⁵ M CCP; □, 10⁻⁴ M CCP; △, 2 × 10⁻⁴ M CCP.*

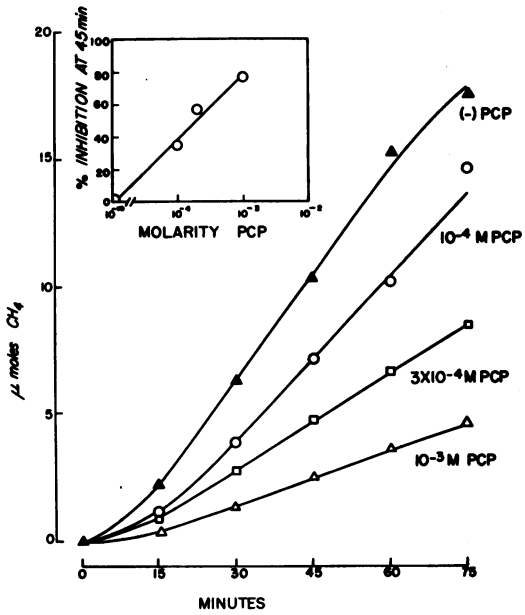

FIG. 11. *Effect of pentachlorophenol (PCP) on methane production by cell-free extracts. Cell-free extract (37 mg of protein suspended in 1 ml of 50 mM potassium TES, pH 7.4) was placed in the main compartment of a Warburg flask, and 10 μmoles of ATP and PCP in 0.5 ml of buffer were placed in the side arm. Flasks were treated as described in the legend to Fig. 9. Symbols: ▲, no PCP; ○, 10⁻⁴ M PCP; □, 3 × 10⁻⁴ M PCP; △, 10⁻³ M PCP.*

phosphorylation in cell-free extracts, the values were so low that the experiments were discontinued.

Since cell extracts showed no (or virtually no) ATP formation, it was decided to examine the factors that might affect ATP pool levels in whole cells. It was rather surprising to find that inhibition of methane production by air was a relatively slow process. When cells are actively metabolizing hydrogen, apparently sufficient reducing power is maintained even in the presence of small amounts of oxygen. This result stands in contrast to the sensitivity of cells exposed to air in the absence of hydrogen, as shown by the lag in methane production from cells which have been exposed to air during harvesting. When air was added, it was noted that ATP pool levels decreased at a rate proportional to the decrease in methane production; this result would be expected if ATP were generated during methane synthesis. In the presence of chloroform, methane production quickly ceased, as noted previously (17), and ATP pool levels dropped correspondingly.

In mitochondria, it is postulated that DNP, CCP, and PCP uncouple oxidative phosphorylation by causing hydrolysis of the hypothetical nonphosphorylated high-energy intermediate which is believed to mediate energy transfer between the primary energy conserving reaction and the ultimate synthesis of ATP. Uncouplers have no direct effect on electron transport between the carriers, though some do have separate actions on electron transport (e.g., dicumarol). If these three compounds, DNP, CCP, and PCP, had a similar uncoupling effect on the methane bacterium, one might have expected the ATP levels in whole cells to decrease and methane production to be unaffected or increased. Both ATP levels and methane production decreased by proportional amounts. As yet, there is no satisfactory explanation for these results, but three possibilities may be considered.

(i) The compounds may effect the transport of metabolites across the cell membrane (5). Though this explanation may have some validity, additional factors must be involved, since it has been demonstrated that these compounds also inhibit

methane production by cell extracts for which we have no evidence of any membrane-containing particulate structures with permeability barrier properties.

(ii) The compounds might act as inhibitors of electron transport, which would be consistent with the whole-cell experiments and with the cell-free extract experiments. It should be noted that PCP and CCP are both halogenated compounds, possibly suggesting a similar method of action to that of chloroform. DNP inhibition can be overcome in time, suggesting DNP is slowly reduced by the methane-producing system. However, CCP and PCP inhibitions are not overcome with time but appear to remain effective; if they are acting as electron transfer inhibitors, they are not doing so by acting as electron acceptors.

(iii) It might be envisaged that ATP levels control methane production, i.e., factors altering one will also alter the other. If one of the steps in methane production is energy-linked, requiring energy from ATP to overcome an energetically unfavorable equilibrium, and if CCP or PCP uncouple this reaction, they would at the same time inhibit methane production in whole cells or extracts.

This third possibility would fit with a number of formerly unexplained observations. It has been shown that the viologen dyes at very low concentrations prevent methane production by whole cells and by extracts (16). If the dye acts by short-circuiting electrons around an energy conservation site, then it would be impossible to make ATP in whole cells, and impossible to drive an ATP-requiring, energy-linked reaction. The finding that the rate of methane synthesis is proportional to the levels of ATP found in the cell could reflect ATP generation during methane synthesis. Such a mechanism could also explain why conditions have not yet been found in which methane production rates can be dissociated from ATP pool levels.

The possibility should not be excluded that, in *Methanobacterium*, the relevant factor controlling methane production could be AMP, not ATP. The rate of methane formation is inversely proportional to AMP levels. It has been noted previously that addition of AMP to cell extracts inhibits methane formation (18). A recent study has shown that the amount of ATP required for methane formation from methyl cobalamin in extracts is not a stoichiometric ratio of 1 mole of ATP per mole of methane formed (Roberton and Wolfe, Biochim. Biophys. Acta, in press).

ACKNOWLEDGMENT

This investigation was supported by National Science Foundation grant GB-4481 and by Public Health Service grant SW0045.

We thank M. P. Bryant and Richard Crabill for kindly supplying cultures of the organism.

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