JOURNAL OF BACTERIOLOGY, Dec. 1979, p. 1081-1089
0021-9193/79/12-1081/09$02.00/0

Vol. 140, No. 3

Chemiosmotic Coupling in *Methanobacterium thermoautotrophicum*: Hydrogen-Dependent Adenosine 5'-Triphosphate Synthesis by Subcellular Particles

HANS J. DODDEMA,[1]* CHRIS VAN DER DRIFT,[1] GODFRIED D. VOGELS,[1] AND MARTEN VEENHUIS[2]

Department of Microbiology, Faculty of Science, University of Nijmegen, 6525 ED Nijmegen, The Netherlands,[1] and Department of Electron Microscopy, Biological Center, University of Groningen, 9751 NN Haren, The Netherlands[2]

Received for publication 17 September 1979

Hydrogenase and the adenosine 5'-triphosphate (ATP) synthetase complex, two enzymes essential in ATP generation in *Methanobacterium thermoautotrophicum*, were localized in internal membrane systems as shown by cytochemical techniques. Membrane vesicles from this organism possessed hydrogenase and adenosine triphosphatase (ATPase) activity and synthesized ATP driven by hydrogen oxidation or a potassium gradient. ATP synthesis depended on anaerobic conditions and could be inhibited in membrane vesicles by uncouplers, nigericin, or the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide. The presence of an adenosine 5'-diphosphate-ATP translocase was postulated. With fluorescent dyes, a membrane potential and pH gradient were demonstrated.

In microorganisms the proton motive force $\Delta\tilde{\mu}_{H^+}$ across the cell membrane consists of two components: a membrane potential $\Delta\psi$ and a proton gradient ΔpH (27). The proton motive force may be built up by an oxidative proton-extrusing electron transport chain (11) or by light-driven proton extrusion, as in *Halobacterium* (13, 31) or photosynthetic bacteria (15); this force can be used to drive ATP synthesis and transport processes. Alternatively ATP may be hydrolyzed to produce the proton motive force needed for transport processes.

ATP synthesis in the absence of oxygen can be coupled to an electron transport chain using nitrate, sulfate, fumarate, or carbon dioxide as terminal electron acceptors (36).

Roberton and Wolfe (33) showed that the ATP concentration in cells of *Methanobacterium* strain MoH increased during the conversion of H_2 and CO_2 to CH_4. Uncouplers inhibited CH_4 production, and the ATP level decreased. We showed ATP synthesis in whole cells due to an artificially imposed proton motive force (6). Uncouplers stimulated ATP synthesis. This may be explained by assuming that ATP synthesis and hydrogen oxidation are located on internal membranes (46).

Internal membrane systems have been described for many genera of microorganisms. Apart from the mesosomes with unknown function (10), systems have been described for various bacteria: the denitrifying bacteria (29), the phototrophs (30), methane oxidizers (5), *Acetobacterium woodii* (25), and the methanogenic bacteria (20, 47). The involvement of internal membranes in energy conservation has been reported for some of these genera (8, 19, 26), but for the methanogenic bacteria no evidence for such involvement existed. As mentioned, whole cells of *M. thermoautotrophicum* synthesized ATP because of an artificially imposed proton motive force (6). This ATP synthesis was not inhibited by the adenosine triphosphatase (ATPase) inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) and was enhanced by the uncouplers carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) and 2,4-dinitrophenol (DNP) (6). The anomalous effect of these compounds can be explained by two mechanisms: (i) the cell wall or an outer membrane excludes these compounds from the ATPase, and (ii) the energy generating system is localized internal to the cytoplasmic membrane. The cell walls of methanogens have unusual properties (17). *M. thermoautotrophicum* walls contain a pseudomurein layer (16). To further test the aforementioned hypotheses, the ultrastructures of whole cells and membrane vesicles were studied. Also ATP synthesis by subcellular particles was studied to avoid interference by the above-mentioned mechanisms. The study also tries to show that ATP synthesis occurs according to chemios-

motic principles in *M. thermoautotrophicum*, an organism from the unique and phylogenetically ancient group of methanogens (45).

MATERIALS AND METHODS

Organism. *M. thermoautotrophicum* strain ΔH was a gift of R. S. Wolfe, Urbana, Ill. Cultures were grown in a 12-liter fermentor in a medium containing (in millimolar concentrations): $NaHCO_3$, 40; NH_4Cl, 23; Na_2HPO_4, 3.3; cysteine, 1.4; Na_2S, 1; KCl, 1.9; $(NH_4)_2SO_4$, 1.3; $MgSO_4$, 0.2; $CaCl_2$, 0.06; $FeSO_4$, 0.03; (in micromolar concentrations) $MnSO_4$, 2; H_3BO_3, 1.6; $ZnSO_4$, 1.5; $CoSO_4$, $CuSO_4$, and $NaMoO_4$, 0.4; and $AlKSO_4$, 0.2. The culture was gassed with H_2-CO_2 (80 and 20% [vol/vol], respectively). The pH during growth was between 7 and 7.8, and the temperature was maintained at 65°C. Cells were harvested under N_2 at the end of exponential growth in a Sharples continuous centrifuge. The yield was 1 mg (dry weight) of cells per ml of culture. Cells were stored anaerobically under H_2 at −80°C.

Membrane preparation. Membranes were prepared as described previously (6) or by the method of Michels and Konings (26) and stored anaerobically under H_2 at −80°C. Membranes prepared by either method showed similar properties. For some tests, membranes were further purified by layering the membrane fraction on top of a 5% Ficoll solution. After 1 h of centrifugation at 80,000 × *g*, the membranes were collected from the upper layer. For electron microscopy of membrane vesicles, the membranes were purified by centrifugation at 50,000 × *g* until a clear supernatant was obtained. This supernatant was charged on top of a discontinuous Ficoll gradient of 6, 3, and 1.5% Ficoll. After 1 h of centrifugation at 80,000 × *g*, the membranes were collected from the interphase between 6 and 3% Ficoll. These membranes were diluted in 0.1 M bicine (pH 8.3), containing 1 mM $MgCl_2$, and centrifuged for 3 h at 150,000 × *g*. The pellet was resuspended in bicine buffer.

Fluorescence measurements. Changes in the proton motive force were determined from quenching of the fluorescence of 3,3′-dipropyl-2,2′-thiacarbocyanine iodide [Di-S-C_3(5)] (41), analinenaphthalenesulfonic acid, or 9-aminoacridine. Changes in the fluorescence were determined in an Aminco-Bowman spectrophotofluorometer.

Test procedures. ATP synthesis in whole cells, potassium concentration (by flame photometry or a potassium electrode), internal volume and pH, and proton uptake were determined as described by Doddema et al. (6). Vesicles were centrifuged at 150,000 × *g* for 3 h in all procedures.

ATP synthesis by membrane particles was determined in 5 ml of 100 mM bicine buffer, pH 8.3 (at 20°C), containing 5 mM $MgCl_2$, 2.5 mM inorganic phosphate, and 0.625 mM ADP (unless otherwise stated). The test was performed at 65°C under an atmosphere of N_2 or N_2-H_2 (30 and 70% [vol/vol], respectively). Samples were withdrawn from the reaction vessel with a glass syringe via a rubber septum and transferred to a test tube at 100°C as rapidly as possible. After 30 s, the test tube was put into an ice-water bath until the ATP determination. ATP was determined with the firefly luciferin-luciferase system. ADP and AMP were measured after conversion to ATP (3).

Degradation of cell walls. The following enzymes were used for attempts to degrade the cell walls: pronase; phospholipase; α-amylase; papain; lysozyme (also in combination with ethylenediaminetetraacetic acid or Brij or sodium dodecyl sulfate); cellulase; chitinase; trypsin; chymotrypsin; lipase; dextranase; pepsin; rennin; pancreatin; zymolyase; hélicase; pectate lyase; and cathepsin D. Cells were incubated in an appropriate buffer according to the supplier but were further treated according to Witholt et al. (44). Lysis was also tried by repeated freezing and thawing and by freeze-drying followed by dissolving.

Hydrogenase assay. Hydrogenase was routinely assayed in type 220 cuvettes (Vapotherm b.v., Alphen aan de Rijn, Holland) closed with rubber stoppers. The cuvettes were evacuated five times and filled with H_2. Then 2.7 ml of an anaerobic hydrogen-saturated 0.1 M $NaPO_4$ buffer (pH 7.5) with 3 mM methylviologen was added to the cuvette by means of a glass syringe. The cuvette was evacuated once more and filled with hydrogen. A series of 10 cuvettes was prepared at the same time. The cuvettes were incubated at 65°C. To start the reaction, 0.3 ml of an anaerobic enzyme solution with a protein content of about 1.5 mg ml^{-1} was added. The change in extinction at 602 nm was read at 65°C in a Cary 118 spectrophotometer.

Protein. The method of Lowry et al. (23) was used to determine the protein content with bovine serum albumin as standard.

Dry weight. Dry weight was determined by drying cells at 60°C in vacuo.

Electron microscopy. After prefixation in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 20 min, whole cells were fixed with 2% OsO_4 in 0.1 M Veronal-acetate buffer (pH 7.2) and poststained with 3% uranylacetate in water for 10 min and lead citrate according to the method of Venable and Coggeshall (40).

For thin sectioning, cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min and postfixed with a mixture of 1% OsO_4 and 5% $K_2Cr_2O_7$ in the same buffer for 2 h. Alternatively, glutaraldehyde was added to the culture medium to a concentration of 3%; after 5 min of incubation at 65°C, cells were centrifuged. Postfixing was done as described above, or the technique of Kellenberger et al. (18) was used.

Hydrogenase was stained with Hatchett's brown (34), or a series of tetrazolium salts (1). ATPase was stained according to the method of Rechardt and Kokko (32). After routine fixation or cytochemical staining, cells were embedded in Epon or Araldite (Fluka, Buchs, Switzerland), and ultrathin sections were cut with an LKB ultratome 1. Alternatively, cells were cut (without embedding) by cryo-ultramicrotomy and stained with uranylacetate. Freeze-etching of cells was performed with a Balzer unit by the technique of Moor (28). Sections were examined with a Philips 201 or 300 electron microscope.

Fatty acids. Fatty acids in whole cells were determined by analysis of their methylesters by gas-liquid chromatography on a Pye series 104 gas chromatograph (42).

Quinones. Quinones were determined by the

method of Crane and Dilley (4).

Chemicals. Gases were obtained from Hoek Loos, Schiedam, Holland. To remove all traces of oxygen, the gases containing H_2 were passed over a catalyst (BASF R 0-20) at room temperature; other gases were passed over a BASF R 3-11 catalyst at 150°C.

DCCD was obtained from Aldrich Europe, Beerse, Belgium; CCCP was from Calbiochem, La Jolla, Calif.; valinomycin, DNase, ADP (less than 0.2% ATP), ATP, and myokinase were from Boehringer Mannheim, Mannheim, West Germany. [^{3}H]inulin was from the Radiochemical Centre, Amersham, England, and 5,5-[^{14}C]dimethyl-2,4-oxazolidinedione and ^3H_2O were from New England Nuclear Corp., Boston, Mass. Pronase, phospholipase, α-amylase, papain, and lysozyme were supplied by Boehringer Mannheim; cellulase, chitinase, trypsin, chymotrypsin, triphenyltetrazolium chloride, and α-cetyl citric acid were from E. Merck AG, Darmstadt, West Germany; lipase, dextranase, pepsin, rennin, and atractyloside were from Sigma Chemical Co., St. Louis, Mo.; pancreatin was from Serva, Heidelberg, West Germany; zymolyase was from Kirin Brewery, Takasaki, Japan; hélicase was from Industrie Biologique Française, Gennevilliers, France. 8-Azido-ADP was a gift from R. Wagenvoort, Amsterdam, Holland, pectate lyase was a gift from F. Rombouts, Wageningen, Holland, and cathepsin D was a gift from A. Bouma, Groningen, Holland. Tetranitroblue tetrazolium chloride was obtained from Serva; thiocarbamylnitroblue tetrazolium and 2-(2′-benzothiazolyl)-styryl-3-(4′-phthalhydrazidyl)tetrazolium chloride were from Polyscience Inc., Warrington, Pa. 8-Anilino-1-naphthalene sulfonic acid was obtained from Sigma; Di-S-C_3(5) was a gift from A. Waggoner, Amherst College, Mass.; and 9-amino-acridine was obtained from Fluka, Buchs, Switzerland.

RESULTS

Ultrastructure and cytochemical stains. Positive staining of whole cells showed an additional layer outside the pseudomurein layer (Fig. 1). This layer formed a sheath enveloping a series of cells. The layer is also observed in thin sections of *M. thermoautotrophicum* cells (Fig. 2).

Attempts to degrade the cell wall with the lytic enzymes mentioned in Materials and Methods, to obtain spheroplasts, have failed, even after a mild osmotic shock was applied to facilitate passage of the lytic enzymes through the outer layer of the cell wall (44). Therefore it was impossible to determine the influence of the sheath or the pseudomurein layer on the effect of the uncouplers and DCCD.

Thin sections of cells prefixed in glutaraldehyde at room temperature (Fig. 3 and 4) show the internal membrane system in this organism as reported before (20, 47). Fixation at room temperature may result in mesosome-like structures as artifacts; to avoid this, cells were fixed with glutaraldehyde in their growth medium at 65°C (Fig. 5). Moreover, cryosections of unfixed cells and freeze-fracture replicas were prepared from cells frozen immediately from the growth medium. All three methods showed the presence of internal membranes, probably as invaginations from the cytoplasmic membrane.

After incubation of washed cell suspensions under H_2 with tetrazolium salts or ferricyanide as electron acceptors for the hydrogenase, the reaction product was found exclusively on the invaginating membranes (Fig. 6–10). Especially the membranes near the cytoplasm were heavily stained (Fig. 8, 9, 10). No stain was deposited when cells were incubated under N_2 or when O_2 was present together with H_2. With tetrazolium salts, granular formazan deposits could be observed in the cells even with the phase-contrast light microscope. No stain was ever deposited on the cytoplasmic membrane. To exclude the possibility that this was due to improper staining conditions, cells of *Hyphomicrobium* were stained; in these cells a formazan deposit was observed on the cytoplasmic membrane (Y. Meiberg, unpublished data). It was concluded that all hydrogenase activity was localized on the internal membranes.

Staining the ATPase resulted also in stain deposits of lead phosphate on the internal membranes (Fig. 11). Some stain deposit was also observed in the cytoplasm and on the cytoplasmic membrane, possibly due to the activity of ATPase or other ATP-hydrolyzing enzymes or to the deposit of lead carbonate. It was concluded that at least part of the ATPase is localized on the internal membranes.

With the localization on the internal membranes of hydrogenase and part of the ATPase, both enzymes involved in energy conservation, it appeared that ATP synthesis might occur beyond the cytoplasmic membrane on specialized organelles.

Freeze-fracturing of cells. Whole cells were freeze-fractured to determine whether the orientation of the membrane, i.e., whether it was inside or right-side out, could be decided from the presence of proteins and to look for the continuity of the internal membranes with the cytoplasmic membrane. However, cells did not split over the membrane but through the cytoplasm. Chemical analysis of the membranes showed the presence of small amounts of $C_{16:0}$, $C_{14:0}$, and $C_{18:0}$ fatty acids with minor amounts of $C_{13:0}$, $C_{15:0}$, and $C_{17:0}$ and hardly any branched-chain fatty acids. These data confirm previous data (24), and it seems unlikely that the presence of these fatty acids prohibits splitting of the membrane. The squalenes and phytanyl glycerol ethers present in the cell membranes (24, 38) are only slightly different from those found in halobacteria; *Halobacterium* membranes could be split by freeze-fracturing (13), so it remains an

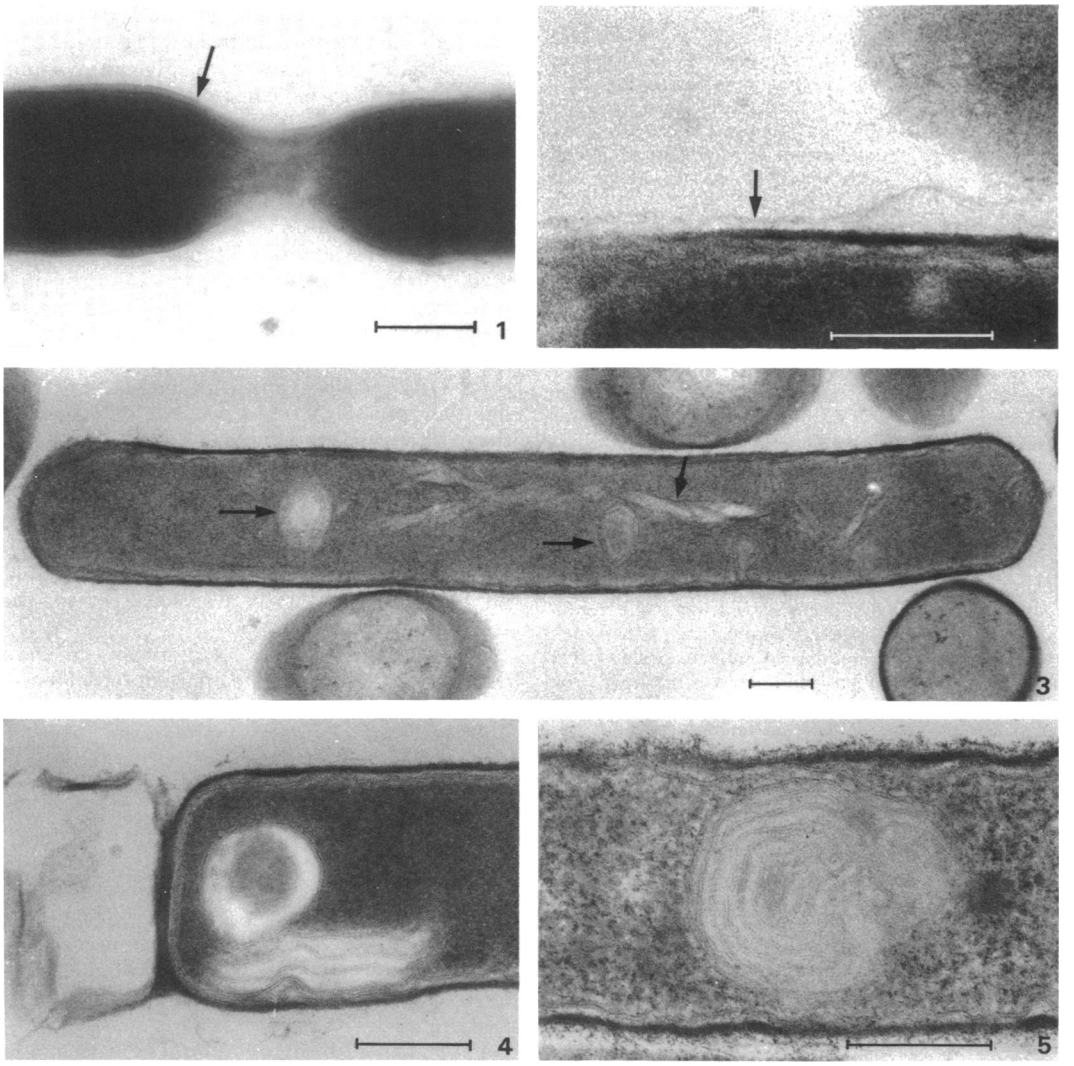

FIG. 1–5. *All micrographs show M. thermoautotrophicum strain ΔH, grown as described in Materials and Methods and fixed with glutaraldehyde and OsO_4-$K_2Cr_2O_7$. The markers represent 0.2 μm, unless otherwise indicated.*

FIG. 1. *Detail of an intact whole cell, fixed with OsO_4 and poststained with uranylacetate and lead citrate to demonstrate the presence of a sheath around the cells (arrow).*

FIG. 2. *Detail of a thin section of a cell, fixed with glutaraldehyde at 65°C before postfixation, to show the sheath (arrow) around the cell.*

FIG. 3. *Thin section of a cell, fixed with glutaraldehyde at room temperature before postfixation. Membranous structures can be observed.*

FIG. 4 and 5. *Magnification of cells fixed with OsO_4 by the method of Kellenberger et al. (17) (Fig. 4) and prefixed with glutaraldehyde at 65°C before postfixation (Fig. 5) to show different membranous structures inside the cells.*

open question why membranes of *M. thermoautotrophicum* were not split.

Membrane vesicles. Subcellular particles were prepared for studying chemiosmotic coupling in *M. thermoautotrophicum*.

Physical properties of the membrane particles. Negatively stained preparations and thin sections of membrane preparations showed closed membrane vesicles of about 50 nm in diameter which, even after extensive purification, were still contaminated with some cell walls and many small particles with diameters of 10

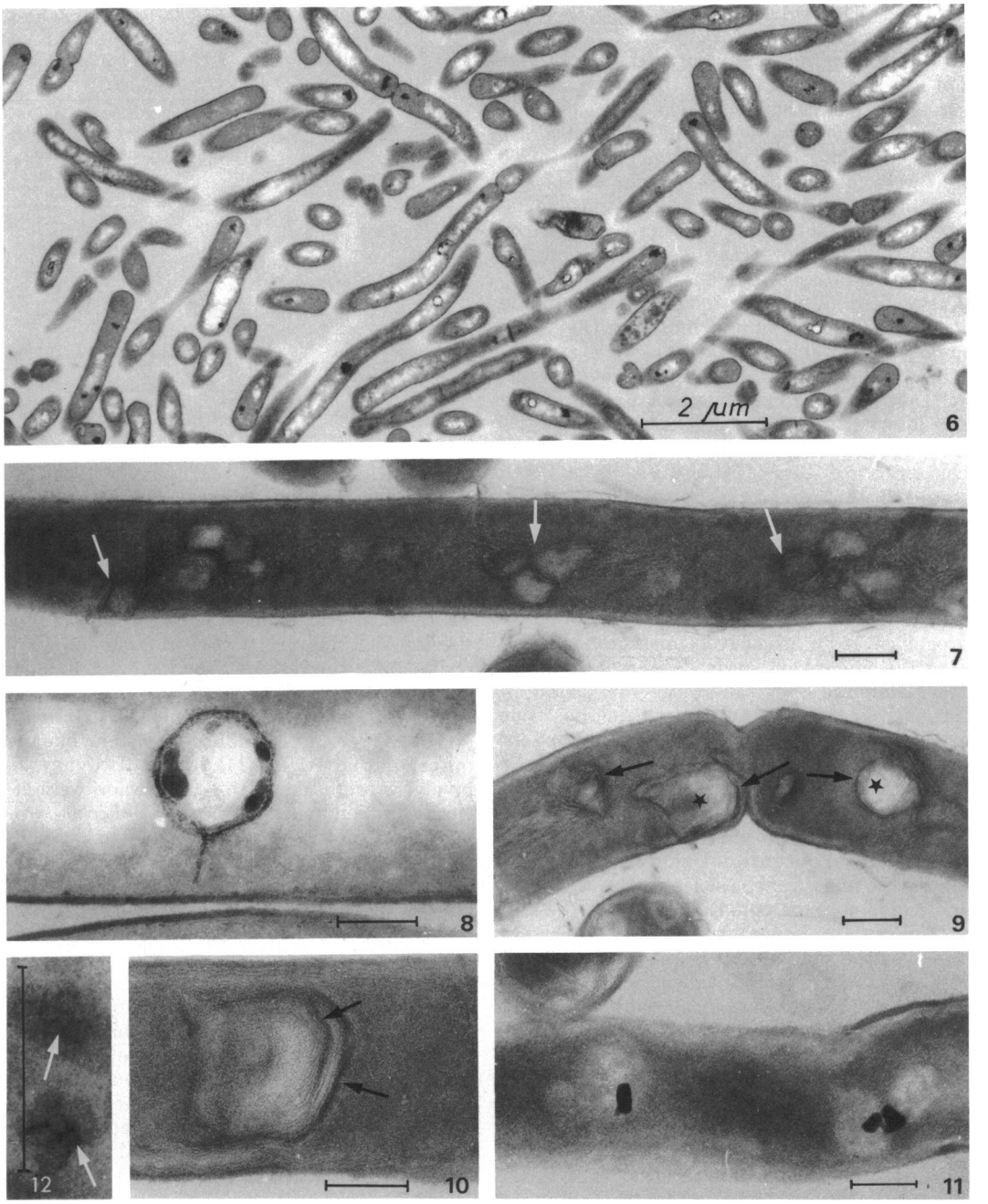

FIG. 6–12. *See legend to Fig. 1–5 for details.*

FIG. 6. *Survey of cells after incubation with ferricyanide to demonstrate hydrogenase activity. The reaction product was amplified with diaminobenzidine. The reaction product was exclusively located on internal cell membranes.*

FIG. 7. *Demonstration of hydrogenase activity after incubation with BSPT [2-(2'-benzothiazolyl)-styryl-3-(4'-phtalhydrazidyl)tetrazolium chloride]. As after incubation with ferricyanide (Fig. 6), the reaction product was exclusively located on the internal cell membranes.*

FIG. 8. *Magnification of Fig. 6 to show that the reaction product is mainly located on the membranes facing the cytoplasm.*

FIG. 9 and 10. *Thin section of cells after incubation with BSPT to demonstrate the localization of hydrogenase activity. As in Fig. 8, the reaction products are mainly located on the membranes facing the cytoplasm (arrows), leaving other invaginating membranes unstained (asterisk).*

FIG. 11. *Thin section of a cell tested for ATPase activity. The reaction product is located mainly inside the internal membrane structures.*

FIG. 12. *Hexagonal particles present in BSPT-stained vesicle preparations.*

to 15 nm (Fig. 12). These particles usually were not attached to the vesicles; they were also observed in association with the internal membrane system in cryosections of whole cells. Also some larger particles with diameters up to 250 nm were observed. Freeze-fracturing of the membrane vesicles was not possible since membranes were not split. Therefore no electron microscopic evidence was obtained regarding the orientation of the membranes.

Biochemical properties of the membrane vesicles. The internal volume of the crude membrane preparation was 20 μl per mg of protein. Assuming that there was an equal internal K^+ concentration in all vesicles, we found that the internal potassium concentration was 230 mM. The internal pH was 7.7, and the internal concentrations of AMP, ADP, ATP, and PO_4 were 180, 17, 2, and 1.4 μM, respectively; cofactors F_{420} and F_{342} were also present in low quantities. The internal K^+ concentration and pH resembled the values found in whole cells (6). The membranes showed ATPase activity. Although electron microscopy had shown the hydrogenase to be exclusively membrane associated, the amount of hydrogenase associated with the membrane preparation was 18%, based on the average of the total activity in the crude extract, whereas the 150,000 × *g* supernatant contained 75% on the average. Obviously the hydrogenase dissolved readily from the membrane.

ATP synthesis by membrane vesicles due to membrane potential. Under anaerobic conditions the membrane vesicles synthesized ATP due to a membrane potential (Table 1); the rate of ATP synthesis varied from 0.4 to 1.5 nmol min^{-1} mg $protein^{-1}$. This value corresponds well with that found for *Escherichia coli* vesicles (39). The ATP synthesis could be inhibited by increasing concentrations of K^+ in the buffer. Valinomycin did not enhance ATP synthesis. Obviously the membranes do not need an added potassium ionophore to create a membrane potential. Membranes showed a temperature-dependent potassium leak: at 4°C no potassium was lost during 30 min of incubation; at 37°C the internal potassium concentration had decreased from 230 to 190 mM without 10^{-5} M valinomycin and to 180 mM with 10^{-5} M valinomycin; and at 65°C it decreased to 160 mM.

Effect of inhibitors. ATP synthesis could be inhibited by 0.1 mM DCCD, 0.1 mM CCCP, 1 mM DNP, or 0.01 mM nigericin (Table 1). In membrane vesicles these compounds affect ATP synthesis in a normal way.

Storage of membranes. Membrane preparations retained some ATPase activity due to $\Delta\psi$ after 16 months of aerobic storage at −80°C.

TABLE 1. *ATP synthesis due to a potassium gradient or the presence of* H_2[a]

Gas phase	K^+ concn (mM)	Inhibitor (concn, mM)	ATP synthesis (nmol min^{-1} mg^{-1})
N_2	—[b]	—	1.7
N_2	10	—	1.0
N_2	100	—	0.8
N_2	300	—	0
N_2	—	DNP (1)	1.3
N_2	—	DCCD (0.1)	0.8
N_2	—	CCCP (0.1)	0
H_2	300	—	2.8
H_2	300	DNP (1)	0
H_2	300	nigericin (0.01)	0
H_2	300	DCCD (0.01)	0
H_2	300	CCCP (0.01)	0

[a] A 0.2-ml portion of a vesicle solution containing about 5 mg of protein was added to the reaction mixture as described in Materials and Methods.
[b] —, Not present.

Effect of translocase inhibitors. To synthesize ATP due to a potassium gradient ([K^+] inside high), the vesicles must have a right-side-out orientation (ATP synthetase inside). Since ATP synthesis strictly depended on externally added ADP, a specific transport system, like the mitochondrial ADP-ATP translocase, may be involved. Inhibitors of this translocase were effective in membrane vesicles of *M. thermoautotrophicum*: atractyloside (50 μM), 8-azido-ADP (0.4 mM), and α-cetyl citric acid (20 μM) completely inhibited ATP synthesis driven by the membrane potential. These results strongly suggest the presence of an ADP-ATP translocase in *M. thermoautotrophicum*.

Hydrogen-dependent ATP synthesis. When membrane vesicles were incubated under N_2 with 300 mM KCl added to the buffer to prevent ATP synthesis due to the potassium gradient, no ATP synthesis occurred (Table 1). However, as soon as H_2 was introduced into the reaction vessel, ATP synthesis started. This synthesis could be inhibited by 0.1 mM CCCP, 0.1 mM DCCD, 0.01 mM nigericin, or 1 mM DNP (Table 1). ATP synthesis did not occur without added ADP, with boiled membranes, or when O_2 was present. The specific activity of phosphorylation was between 1 and 8 nmol min^{-1} mg $protein^{-1}$. This value compares well with that found for hydrogen-dependent ATP synthesis in *Alcaligenes eutrophus* (14).

ATP synthesis due to a ΔpH created by the hydrogenase activity may be expected in both right-side-out and inside-out vesicles as H_2 freely diffuses through the membrane. Since the translocase inhibitor atractyloside only partially

inhibited ATP synthesis with H_2—at 50 μM atractyloside between 20 and 80% inhibition occurred with different membrane preparations—it was concluded that both types of vesicles were present.

ATP synthesis due to an artificially imposed proton motive force. In contrast to whole cells, membrane vesicles did not synthesize ATP due to an artificially imposed proton motive force created by an acid or base pH shift aerobically or anaerobically. Also, the addition of valinomycin to membranes did not lead to ATP synthesis under aerobic conditions; as mentioned, no valinomycin was needed for ATP synthesis under anaerobic conditions. ATP synthesis strictly depended on anaerobic conditions, in contrast to ATP hydrolysis by the membranes and ATP synthesis in whole cells.

Measurement of the proton motive force with fluorescent dyes. To demonstrate that ATP synthesis coincided with the presence of a membrane potential ($\Delta\psi$) due to a potassium gradient and a ΔpH created by hydrogenase activity, $\Delta\psi$ and ΔpH were measured with the fluorescent dyes Di-S-C_3(5) and 9-aminoacridine, respectively.

$\Delta\psi$ was demonstrated by using Di-S-C_3(5) fluorescence quenching in both whole cells and membrane vesicles. In whole cells hyperpolarization was induced by the addition of valinomycin, whereas in vesicles no valinomycin has to be added. This agrees with the dependence on valinomycin for ATP synthesis and potassium leakage in whole cells, but not in vesicles. Both cells and vesicles could be deenergized by the addition of 0.4 mM nigericin, but not by gramicidin.

In vesicles the magnitude of the membrane potential depended on the external potassium concentration. Anaerobic conditions were no prerequisite for the formation of $\Delta\psi$.

With 9-aminoacridine a ΔpH was measured in vesicles. Fluorescence quenching under H_2 was about 10%, whereas it was 3% under N_2. The fluorescence quenching of 9-aminoacridine indicates a ΔpH inside acid (2), indicating the presence of inside-out vesicles. With analine-naphthalenesulfonic acid no meaningful results could be obtained since fluorescence did not change after deenergization with nigericin or gramicidin. No proton movements could be measured with a pH probe as direct evidence for a ΔpH.

Effects of trypsin and Mg^{2+}. Membranes treated with trypsin lost the ability to synthesize ATP, but hydrogenase activity was hardly influenced. ATP synthesis depended on the presence of Mg^{2+}. These results correspond to those found for ATP hydrolysis (6).

Adenylate kinase activity. Adenylate kinase presumably is not involved in the ATP synthesis found; a commercial preparation of adenylate kinase was not inhibited by nigericin, CCCP, DCCD, or DNP. Moreover, the activity of adenylate kinase was stimulated by an increase of the K^+ concentration in the buffer.

DISCUSSION

The hydrogenase of *M. thermoautotrophicum* was stained with a series of different electron acceptors and under various conditions to minimize possible misinterpretation of the results (10). Hydrogenase appears to be exclusively localized on the invaginations of the cytoplasmic membrane. Also, part of the ATPase is localized on these membranes. *M. thermoautotrophicum* appears to have developed specialized organelles for ATP production, as also occurs in other genera of bacteria (8, 19, 26). A comparison with the mitochondria of eucaryotic organisms is appealing both in structure and function: the internal membranes sometimes occur as a highly convoluted system and there is a strong indication of the presence of an ADP-ATP translocase. Such translocases have been described twice in bacterial systems (12, 43), but in *Rickettsia* they are reportedly insensitive for mitochondrial translocase inhibitors (43).

The temperature-dependent potassium permeability of the membrane vesicles of *M. thermoautotrophicum* may be due to the aberrant lipid composition of the methanogens (24, 38), which resembles the lipid composition in halophilic organisms (38). Vesicles of *H. halobium* also showed a K^+ permeability higher than that of whole cells (21). The rate of K^+ leakage of *M. thermoautotrophicum* compares with that of the *Halobacterium* membrane vesicles (21).

Uncouplers stimulated ATP synthesis in whole cells. Electron microscopy showed that both mechanisms can explain these anomalous results, i.e., interference of the cell wall or the localization of the ATP synthetase beyond the cytoplasmic membrane, or both.

Freeze-fracturing of membrane vesicles did not give direct evidence of the orientation of the vesicles since the membranes would not split. However, the ATP synthesis due to $\Delta\psi$ and the demonstration of $\Delta\psi$ with Di-S-C_3(5), with a subsequent increase of fluorescence upon deenergization, and the complete inhibition of ATP synthesis by ADP-ATP translocase inhibitors, all indicate the presence of right-side-out vesicles. The demonstration of a ΔpH with 9-aminoacridine indicates the presence of inside-out vesicles. The only partial inhibition of H_2-dependent ATP synthesis by the translocase inhibitors, i.e., only in the right-side-out but not

in the inside-out vesicles, confirms the presence of both types of vesicles. Separation of the two types of vesicles will be needed to measure $\Delta\psi$ and ΔpH quantitatively.

Anaerobic conditions are required for ATP synthesis by vesicles but not for the response of whole cells to an artificially imposed proton motive force. The membrane potential can be demonstrated under aerobic conditions in both systems; however, aerobic ATP synthesis driven by $\Delta\psi$ was only demonstrated in whole cells. How ATP synthesis is inhibited by O_2 or a high redox potential in vesicles remains unknown; the different sensitivity to O_2 may be caused by the location of the energy-generating system on the internal membrane in whole cells, thus giving protection against rapid oxidation.

The electron carriers and the terminal electron acceptor involved in the oxidation of H_2 by membrane vesicles remain unknown. Coenzyme F_{240} is known to be reduced by the hydrogenases of methanogens, but fluorometric measurements (7) revealed only small amounts of F_{420} or F_{342}. Thauer et al. (36) reported the absence of menaquinone in *M. thermoautotrophicum*. We were unable to detect quinones. The involvement of squalenes as electron sinks as suggested before (38) remains to be proven. Squalenes occur in other groups of procaryotes, e.g., halobacteria (37), cyanobacteria (35), and methane- (2) and methanol-oxidizing (9) microorganisms at levels between 1 and 5 mg g of dry weight^{-1}, and no indication for a role of squalenes as electron acceptors is known in these organisms. Bicarbonate and carbon dioxide, the in vivo electron acceptors, are less likely candidates as electron acceptors in our test system since these compounds were not added and no detectable amounts of CH_4 were formed. A role of the sulfate as electron acceptor seems unlikely since ATP is needed in the reduction to sulfite and no ATP was added. The results lead to the conclusion that ATP synthesis in *M. thermoautotrophicum* may indeed be a process driven by chemiosmotic forces. Further research is now proceeding to measure the magnitude of $\Delta\psi$ and ΔpH and to identify the electron carrier to further support this conclusion.

If methanogens descend from an old diversion of the phylogenetic tree of organisms in one group with the halobacteria (45), where the chemiosmotic principles of ATP synthesis have been very thoroughly demonstrated (22), it must be concluded that ATP synthesis by chemiosmotic principles must have been adopted early in evolution.

ACKNOWLEDGMENTS

We thank J. W. M. Derksen for advice on the positive staining of whole cells, I. Keizer for the preparation of cryosections, and J. Zagers for preparing the electron micrographs.

LITERATURE CITED

1. **Altman, F. P.** 1972. An introduction into the use of tetrazolium salts in quantitative enzyme cytochemistry. Koch-Light Laboratories Ltd., Colnbroak, England.
2. **Bird, C. W., J. M. Lynch, W. Pirt, W. W. Ried, C. J. W. Brooks, and B. S. Middleditch.** 1971. Steroids and squalene in *Methylococcus capsulata* grown on methane. Nature (London) **230**:473–474.
3. **Chapman, A. S., L. Fall, and D. E. Atkinson.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108**:1072–1086.
4. **Crane, F. L., and R. A. Dilley.** 1963. Determination of CoQ (ubiquinone), p. 279–306. *In* D. Glick (ed.), Methods of biochemical analysis, vol. XI. John Wiley & Sons, Inc., New York.
5. **Davies, S. L., and R. Whittenbury.** 1970. Fine structure of methane and other hydrocarbon utilizing bacteria. J. Gen. Microbiol. **61**:227–232.
6. **Doddema, H. J., T. J. Hutten, C. van der Drift, and G. D. Vogels.** 1978. ATP hydrolysis and synthesis by the membrane-bound ATP synthetase complex of *Methanobacterium thermoautotrophicum*. J. Bacteriol. **136**:19–23.
7. **Doddema, H. J., and G. D. Vogels.** 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **36**:752–754.
8. **Eilerman, L. J. M., H. G. Pandit-Hovenkamp, and A. H. Kolk.** 1970. Oxidative phosphorylation in *Azotobacter vinelandii* particles; phosphorylation sites and respiratory control. Biochim. Biophys. Acta **197**:25–30.
9. **Goldberg, I., and I. Schechter.** 1978. Occurrence of squalene in methanol-grown bacteria. J. Bacteriol. **135**: 717–720.
10. **Greenawalt, J. W., and T. L. Whiteside.** 1975. Mesosomes: membranous bacterial organelles. Bacteriol. Rev. **39**:405–463.
11. **Harold, F. M.** 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**:172–230.
12. **Hochman, A., R. Bittan, and C. Carmel.** 1978. Nucleotide translocations across the cytoplasmic membrane in the photosynthetic bacterium *Rhodopseudomonas capsulata*. FEBS Lett. **89**:21–26.
13. **Hwang, S. B., and W. Stoeckenius.** 1977. Purple membrane vesicles: morphology and proton translocation. J. Membr. Biol. **33**:325–350.
14. **Ishaque, H., and M. I. H. Aleem.** 1970. Energy coupling in *Hydrogenomonas eutropha*. Biochim. Biophys. Acta **223**:388–397.
15. **Jones, O. T. G.** 1977. Electron transport and ATP synthesis in the photosynthetic bacteria, p. 151–183. *In* B. A. Haddock and W. A. Hamilton (ed.), Microbial energetics. University Press, Cambridge.
16. **Kandler, O.** 1979. Zellwandstrukturen bei Methan Bakterien zur Evolution der Prokaryoten. Naturwissenschaften **66**:95–105.
17. **Kandler, O., and H. König.** 1978. Chemical composition of the peptidoglycan free cell walls of methanogenic bacteria. Arch. Microbiol. **118**:141–152.
18. **Kellenberger, E., A. Ryter, and J. Sechaud.** 1958. Electron microscopy study of DNA containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleotide in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–676.
19. **Krogman, D. W.** 1973. Photosynthetic reactions and components of thylakoids, p. 80–98. *In* N. G. Carr and B. A. Whitton (ed.), The biology of blue green algae. Blackwell Scientific Publications, Oxford.
20. **Langenberg, K. F., M. P. Bryant, and R. S. Wolfe.** 1968. Hydrogen-oxidizing methane bacteria. II. Electron

microscopy. J. Bacteriol. **95**:1124-1129.
21. **Lanyi, J. K., and K. Hilliker.** 1976. Passive potassium ion permeability of *Halobacterium halobium* cell envelope vesicles. Biochim. Biophys. Acta **448**:181-184.
22. **Lanyi, K.** 1978. Energy conversion in *Halobacterium halobium*. Microbiol. Rev. **42**:682-706.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin reagent. J. Biol. Chem. **193**:265-275.
24. **Makula, R. A., and M. E. Singer.** 1978. Ether containing lipids of methanogenic bacteria. Biochem. Biophys. Res. Commun. **82**:716-722.
25. **Mayer, F., R. Lurz, and S. Schoberth.** 1977. Electron microscopic examination of the hydrogen oxidizing acetate forming anaerobic bacterium *Acetobacterium woodii*. Arch. Microbiol. **115**:207-213.
26. **Michels, P. A. M., and W. N. Konings.** 1978. Structural and functional properties of chromatophores and membrane vesicles from *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **507**:353-368.
27. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41**:445-502.
28. **Moor, H.** 1964. Die Gefrier-Fixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z. Zellforsch. Mikrosk. Anat. **62**:546-580.
29. **Murray, R. G. E., and S. W. Watson.** 1965. Structure of *Nitrocystis oceanus* and comparison with *Nitrosomonas* and *Nitrobacter*. J. Bacteriol. **89**:1594-1609.
30. **Oelze, J., and G. Drews.** 1972. Membranes of photosynthetic bacteria. Biochim. Biophys. Acta **265**:209-239.
31. **Oesterhelt, D., R. Gottschlich, R. Hartmann, and H. Michel.** 1977. Light energy conversion in halobacteria, p. 333-349. *In* B. A. Haddock and W. A. Hamilton (ed.), Microbial energetics. University Press, Cambridge.
32. **Rechardt, L., and A. Kokko.** 1967. Electron microscopic observation on the mitochondrial ATPase in the rat spinal cord. Histochemie **10**:278-286.
33. **Roberton, A. M., and R. S. Wolfe.** 1970. Adenosine triphosphate pools in *Methanobacterium*. J. Bacteriol. **102**:43-51.
34. **Shnitka, T. K. and G. G. Talibi.** 1971. Cytochemical localization by ferricyanide reduction of hydroxy acid oxidase activity in peroxisomes of rat kidney. Histochemie **27**:137-158.
35. **de Souza, N. J., and W. R. Nes.** 1968. Sterols: isolation from a blue green alga. Science **162**:363.
36. **Thauer, R., K. Jungerman, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100-180.
37. **Tornabene, T. G., M. Kates, E. Gelpi, and J. Oro.** 1969. Occurrence of squalene di- and tetrahydro squalenes and vitamin MK_8 in an extremely halophilic bacterium, *Halobacterium cutirubrum*. J. Lipid Res. **10**: 294-303.
38. **Tornabene, T. G., R. S. Wolfe, W. E. Balch, G. Holzer, G. E. Fox, and J. Oro.** 1978. Phytanyl-glycerol ethers and squalenes in the archaebacterium *Methanobacterium thermoautotrophicum*. J. Mol. Evol. **11**:259-266.
39. **Tsuchiya, T., and B. P. Rosen.** 1976. Adenosine 5'-triphosphate synthesis energized by an artificially imposed membrane potential in membrane vesicles of *Escherichia coli*. J. Bacteriol. **127**:154-161.
40. **Venable, L. H., and R. Coggeshall.** 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell. Biol. **25**:407-408.
41. **Waggoner, A.** 1976. Optical probes of membrane potential. J. Membr. Biol. **27**:317-334.
42. **Weerkamp, A., U. Heinen-von Borries, and G. D. Vogels.** 1978. Biochemical and ultrastructural changes in *Staphylococcus aureus* treated with staphylococcin 1580. Antonie van Leeuwenhoek J. Microbiol. Serol. **44**: 35-48.
43. **Winkler, H. H.** 1976. Rickettsial permeability: an ADP/ATP transport system. J. Biol. Chem. **251**:389-396.
44. **Witholt, B., M. Boekhout, M. Brock, J. Kingma, H. van Heerikhuizen, and L. de Ley.** 1976. An efficient and reproducible procedure for the formation of sphaeroplasts from variously grown *Escherichia coli*. Anal. Biochem. **74**:160-170.
45. **Woese, C. R., L. J. Magnum, and G. E. Fox.** 1978. Archaebacteria. J. Mol. Evol. **11**:245-252.
46. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514-541.
47. **Zeikus, J. G., and R. S. Wolfe.** 1973. Fine structure of *Methanobacterium thermoautotrophicum*: effect of growth temperature on morphology and ultrastructure. J. Bacteriol. **113**:461-467.