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Nutrition and Carbon Metabolism of *Methanococcus voltae*

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Methanococcus voltae is a heterotrophic, H_2-oxidizing methanogenic bacterium. In complex medium, this bacterium has a doubling time of 1.2 h at its temperature optimum of 38°C. In defined medium, optimal growth is obtained with 0.75 mM isoleucine, 0.75 mM leucine, 2.5 mM acetate, 5 mM NH_4Cl, 84 mM $MgSO_4$, 0.4 M NaCl, 1 mM $CaCl_2$, 10 μM Fe_2O_3, and 0.2 μM $NiCl_2$. In addition, pantothenate, sodium selenate, and cobalt stimulate growth. Optimal growth is obtained between pH 6.0 and 7.0 with either H_2 or formate as the electron donor. The volatile fatty acids 2-methylbutyrate and isovalerate can substitute for isoleucine and leucine, respectively. Cellular carbon is derived from acetate (31%), isoleucine (22%), leucine (25%), and carbon dioxide (23%). The amino acids and fatty acids are incorporated almost exclusively into protein. A comparison of the incorporation of *U*-^{14}C-amino acids and 1-^{14}C-fatty acids indicated that the fatty acids are degraded during incorporation into cell protein. The distribution of carbon from the amino acids suggests that acetyl coenzyme A is not a major intermediate in the degradation of these compounds. Thus, *M. voltae* may convert isoleucine and leucine to other amino acids by a unique mechanism. The lipid carbon is derived largely from acetate. Thus, the isoprenoid lipids are synthesized de novo from acetate rather than by degradation of leucine. The carbon in the nucleic acids is derived from carbon dioxide (45%), the C-1 of acetate (25%), the C-2 of acetate (22%), and isoleucine and leucine (7%). This labeling pattern is consistent with known biochemical pathways.

Methanogenic bacteria are strict anaerobes which synthesize methane from a limited number of C_1 compounds and acetate. Our understanding of the nutrition, carbon metabolism, and other aspects of the biochemistry of these bacteria has been limited by the difficulty of culturing these organisms. For instance, only a few methanogenic bacteria have been cultivated in defined medium. However, recent improvements in anaerobic techniques for growing methanogens (4, 5) may improve this situation rapidly. On the basis of 16S rRNA sequence homologies, the methanogenic bacteria have been divided into three phylogenetic orders (4). Only three species belonging to the order *Methanococcales* have been isolated. One, *Methanococcus voltae*, has not been described previously in detail. In comparison, the nutrition (17, 18, 31) and morphology (16) and some aspects of the biochemistry (7, 19, 20) of *Methanococcus vannielii* have been examined. In addition, a third member has been described recently (W. J. Jones and M. J. B. Paynter, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, I81, p. 100). To broaden our understanding of this group, we undertook a study of the nutrition and carbon metabolism of *M. voltae*.

M. voltae was isolated from sediment samples taken from Waccasassa estuary in Florida (J. M. Ward, M.S. thesis, University of Florida, Gainesville, 1970). The morphology of this organism is similar to that of *M. vannielii*, although these bacteria differ somewhat in their growth responses to pH. Like the cell wall of *M. vannielii*, the cell wall of *M. voltae* is very fragile. Work with *M. vannielii* has shown that the cells of this organism contain a protein cell wall (16). These two bacteria also have similar isoprenoid lipids (34) and guanine-plus-cytosine contents (4), but they differ in their NaCl requirements (4).

MATERIALS AND METHODS

Bacteria and culture conditions. *M. voltae* strain P.S. (DSM 1537) was obtained from P. H. Smith, University of Florida. The bacteria were grown under a pressurized atmosphere containing H_2 and CO_2 (80:20) (5). Stock cultures were maintained on complex medium. The composition of the complex medium was identical to medium 3 (4), except that 2 g of vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) per liter was substituted for yeast extract and Trypticase and 10 μM sodium selenate was added. The composition of the defined medium was as follows (in

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grams per liter of glass-distilled water): sodium acetate, 1.25; leucine, 0.5; isoleucine, 1.0; pantothenate, 0.005; cysteine, 0.5; nitriloacetic acid, 0.015; resazurin, 0.001; NaCl, 20.0; $NaHCO_3$, 5.0; $MgCl_2 \cdot 6H_2O$, 2.8; $MgSO_4 \cdot 7H_2O$, 3.5; KCl, 0.34; NH_4Cl, 0.25; K_2HPO_4, 0.14; $CaCl_2$, 0.14; $Na_2S \cdot 9H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.005; $Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.001; $CoCl_2 \cdot 6H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; $NiCl_2$, 0.0005; $CuSO_4 \cdot 5H_2O$, 0.0001; $AlK(SO_4) \cdot 2H_2O$, 0.0001; H_3BO_3, 0.0001; $Na_2MoO_4 \cdot 2H_2O$, 0.0001. Anaerobic medium was prepared by boiling the complete medium lacking cysteine and sulfide under a stream of N_2-CO_2 (80:20) gas for 10 s. Solid cysteine was then added, the flask was stoppered, and the medium was dispensed in an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) before autoclaving. Just before inoculation, 1 part of sterile 2.5% $Na_2S \cdot 9H_2O$ was added to 50 parts of medium. The inoculum size was between 2 and 5%. Cultures were grown at 37°C in a reciprocal shaker at 60 rpm.

Additions were made to media by dispensing stock solutions into the culture tubes aerobically. The solutions were made anaerobic during passage of the culture tubes through the air lock of the anaerobic glove box. Anaerobic medium was then added before autoclaving. The potential organic nitrogen sources and pyruvate were incubated as solids in the anaerobic glove box for 24 h. These compounds were then suspended in anaerobic water containing 0.5 g of dithiothreitol per liter and filter sterilized.

In tests to determine the pH range for growth, the following buffers were used: 0.1 M succinate (final pH, 5.5 and 6.0); 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (final pH, 5.7, 5.9, 6.2, 6.4, and 6.7); 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (final pH, 6.7, 6.9, 7.2, 7.4, and 7.7); 0.1 M Tris (final pH, 7.6, 8.1, 8.6, and 8.8); 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (final pH, 7.7, 7.8, and 8.1); and $NaHCO_3$ (final pH, 5.3, 6.0, 6.3, 6.6, 6.8, and 7.1). In each case the sodium concentration was kept constant at each pH by adding appropriate amounts of NaCl. The gas phase used was 2 atmospheres (200 kPa) of H_2-CO_2 (80:20) or N_2-CO_2 (80:20) with 4 g of sodium formate per liter.

Metal-free medium. All glassware and culture tubes were cleaned in concentrated H_2SO_4. Butyl rubber stoppers were washed in a cleaning solution containing 5 g of $NaHCO_3$ per liter and 4 g of EDTA per liter. The glassware and stoppers were rinsed eight times in demineralized water, three times in glass-distilled water, and once in Chelex-treated water (see below). A Chelex-100 column (3 by 23 cm; Bio-Rad Laboratories, Richmond, Calif.) was washed with glass-distilled water until the conductivity of the effluent was 5 mΩ and the pH was 8.0. An additional 20 liters of glass-distilled water was passed through the column, and this was designated Chelex-treated water. Concentrated solutions containing (i) the organic components of the media, (ii) KCl, NaCl, NH_4Cl, and K_2HPO_4, and (iii) $NaHCO_3$ were passed through the Chelex-100 column and diluted with Chelex-treated water to 10 times their final concentrations in the medium. The Chelex-treated solutions and treated water were combined to make Chelex-treated medium. This medium was exposed to gas exchange with N_2 during passage through the air lock to the anaerobic glove box. The medium was reduced by adding Chelex-treated 2.5% $Na_2S \cdot 9H_2O$ prepared by passage through a small, anaerobic column of Chelex-100. The medium was then dispensed into culture tubes containing anaerobic solutions of the metals being tested. Metals were obtained as salts which were more than 99.999% pure from Aldrich Chemical Co., Milwaukee, Wis. Except where noted, the concentrations of the metal salts were as follows: $CaCl_2$, 100 μM; $MgSO_4$, 500 μM; Fe_2O_3, 15 μM; and $NiCl_2$, 2 μM. All other metals and selenium were omitted from this medium. In these experiments cultures were grown at 30°C.

Neutron activation analysis. Bacterial cells were analyzed for metals after neutron activation, as described previously (37). The uncertainties in concentration were one standard deviation, based on counting statistics only.

Analytical procedures. Ammonia was measured as described by Chancy and Marbach (10) after isothermal distillation with saturated $Na_2B_4O_7$ (6). Protein was measured by the biuret procedure (22), except that all volumes were reduced fivefold. The dry weight of whole cells was determined after the cells were washed in defined medium to avoid cell lysis. The cells were resuspended in defined medium and collected by filtration on a polycarbonate membrane (pore size, 0.4 μm; Nuclepore Corp., Pleasanton, Calif.). An equal volume of defined medium was also filtered. The membranes were dried to a constant weight in vacuo over P_2O_5 at room temperature. The dry weight of the cells was determined as the difference between the weight of the membrane from the filtered cell suspension and the weight of the membrane from the filtered defined medium. The absorbance values of the cell suspensions in culture tubes were measured with a Spectronic 20 colorimeter at 660 nm (path length, about 1.5 cm).

Fractionation of whole cells. Whole cells were fractionated by a modification of the procedure of Roberts et al. (26). Washed cells were centrifuged, and the cell pellets were stored at −20°C before use. Each pellet (about 20 mg, dry weight) was suspended in 4 ml of 5% trichloroacetic acid (TCA) and then incubated for 30 min on ice. After centrifugation at 4°C, the supernatant was removed and designated the cold TCA-soluble fraction. The pellet was suspended in 4 ml of 75% ethanol and incubated for 30 min at 45°C. After centrifugation at 23°C, the pellet was suspended in 4 ml of ether-ethanol-water (4:3:1) and incubated for 15 min at 45°C. After centrifugation, the supernatant was combined with the supernatant from the previous centrifugation; 1 part of ether and 1 part of water were added to the pooled supernatants so that two phases were formed. The aqueous phase was designated the alcohol-soluble, ether-insoluble fraction, and the organic phase was designated the alcohol-soluble, ether-soluble fraction. The pellet from the ether-ethanol-water wash was suspended in 4 ml of 5% TCA and then incubated for 30 min in a boiling water bath. After centrifugation at 23°C, the supernatant was removed and designated the hot TCA-soluble fraction. The pellet was suspended in 4 ml of 0.1 M NaOH and designated the residue fraction.

Radiolabeling of whole cells. Cells were grown in 100 ml of defined medium containing 0.2 g of acetate per liter, 0.1 g of isoleucine per liter, and 0.05 g of leucine per liter but no cysteine under 1 atmosphere of H_2-CO_2 (80:20) at 30°C. In some experiments, 0.5 ml of 2-

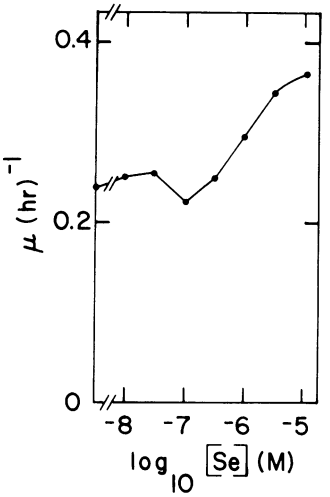

FIG. 1. Selenium requirement for growth. The results are the average growth rates (μ) obtained after three transfers at each concentration of sodium selenate. The complex medium contained 0.2% yeast extract and 0.2% Trypticase instead of Casamino Acids and 0.3 M NaCl.

methylbutyrate per liter and 0.5 ml of isovalerate per liter were substituted for the amino acids. The following amounts of radiolabel were added to parallel cultures: [1-^{14}C]acetate, 4 μCi; [2-^{14}C]acetate, 4 μCi; [*U*-^{14}C]isoleucine, 1.25 μCi; 2-[1-^{14}C]methylbutyrate, 20 μCi; [1-^{14}C]isovalerate, 20 μCi; and $NaH^{14}CO_3$, 25 μCi. Cultures were harvested after 4 days, washed twice with 10 ml of unlabeled medium, and finally suspended in 10 ml of unlabeled medium. (No decrease in the total absorbance of the cell suspension was observed after washing, and less than 3% of the radiolabel in the cells was found in the supernatant fluid of the final wash.) A 0.05-ml portion of each cell suspension was solubilized in 0.3 ml of NCS (Amersham Corp., Arlington Heights, Ill.) and counted with 10 ml of liquid scintillation cocktail (75% [vol/vol] toluene, 25% [vol/vol] Triton X-100, 0.6% [wt/vol] 2,5-diphenyloxazole) in a Beckman model LS 7500 liquid scintillation counter. The radioactivity (in disintegrations per minute) was determined with a [^{14}C]toluene internal standard. The radioactivity in the fractionated cellular components was determined similarly, except that 0.3 ml was counted in 3.0 ml of the liquid scintillation cocktail. On the average, 97% of the radioactivity in the whole cells could be accounted for in the cellular components.

Materials. All of the common reagents used were reagent grade or better. Yeast extract, Casamino Acids, peptone, tryptose, neopeptone, and tryptone were obtained from Difco Laboratories. Trypticase was obtained from BBL Microbiology Systems, Cockeysville, Md. L-Leucine and L-isoleucine were from Nutritional Biochemicals Corp., Cleveland, Ohio. Isovalerate was from Aldrich Chemical Co. 2-Methylbutyrate was from Sigma Chemical Co., St. Louis, Mo. L-[*U*-^{14}C]isoleucine (230 mCi/mmol), L-[*U*-^{14}C]leucine (240 mCi/mmol), and L-[*U*-^{14}C]glutamate (218 mCi/mmol) were obtained from International Chemical and Nuclear Corp., City of Industry, Calif. Sodium [1-^{14}C]acetate (57 mCi/mmol), sodium [2-^{14}C]acetate (55 mCi/mmol), and $Na_2{}^{14}CO_3$ (20 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Sodium [1-^{14}C]isovalerate (4.8 mCi/mmol) was obtained from Research Products International Corp., Elk Grove Village, Ill. Sodium 2-[1-^{14}C]methylbutyrate (3.98 mCi/mmol) was obtained from California Bionuclear Corp., Sun Valley, Calif.

RESULTS

Selenium requirement. In medium 3 of Balch et al. (4) the growth of *M. voltae* was erratic, and the absorbance at the end of growth was generally less than 0.3 cm^{-1}. The addition of 10 μM selenate increased the growth rate by 50% (Fig. 1) and increased the absorbance at the end of growth to at least 0.8 cm^{-1}. Presumably, the contamination of the complex medium by selenium was sufficient to account for the growth observed in the absence of additional selenium (4).

Requirement for branched-chain amino acids. In complex medium, 0.2% yeast extract was required for growth. To determine the nature of the nutrient provided by yeast extract, a wet ash and a dry ash, as well as ether-insoluble and ether-soluble fractions, were prepared. Only the ether-insoluble fraction supported growth. Furthermore, other complex supplements to bacte-

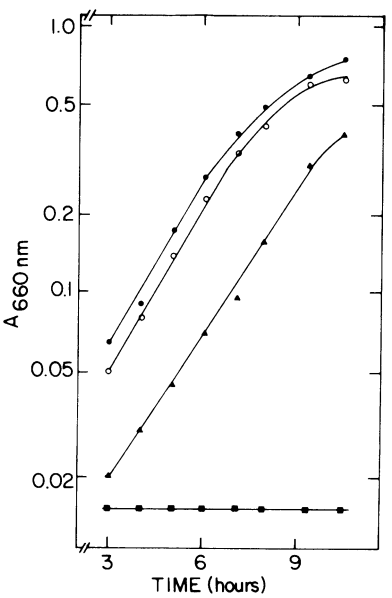

FIG. 2. Amino acids required for growth. Bacteria were grown in defined medium supplemented with 0.1 g of each of 20 common amino acids per liter (●), 1 g of yeast extract per liter (○), 0.1 g of isoleucine per liter and 0.1 g of leucine per liter (▲), and 0.1 g of either isoleucine or leucine per liter (■). A_{660nm}, Absorbance at 660 nm.

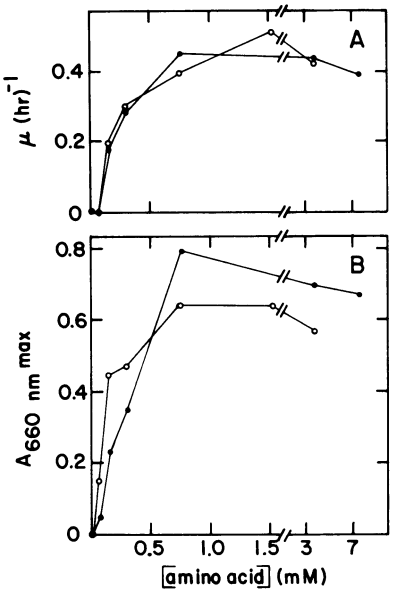

FIG. 3. Titration of isoleucine and leucine. (A) Growth rate (μ) in media containing 0.76 mM leucine and variable concentrations of isoleucine (●) and in media containing 3.8 mM isoleucine and variable concentrations of leucine (○). (B) Final absorbance at 660 nm (A_{660nm}) obtained under the conditions described above.

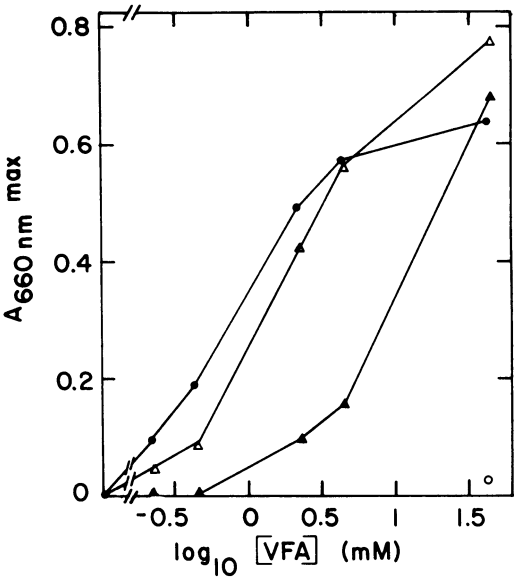

FIG. 4. Substitution of volatile fatty acids (VFA) for the amino acids required for growth. Bacteria were grown either with 7.6 mM isoleucine and variable concentrations of isovalerate (●) and 2-methylbutyrate (▲) or with 7.6 mM leucine and variable concentrations of isovalerate (○) and 2-methylbutyrate (△). A_{660nm}, Absorbance at 660 nm.

rial media were tested for the ability to support growth; both the technical and vitamin-free grades of Casamino Acids substituted for yeast extract. Growth yields were reduced by 60% when equivalent amounts of Trypticase, peptone, and tryptone were used. Very poor growth was obtained with neopeptone. These results suggested that *M. voltae* required some amino acid in yeast extract for growth.

Good growth was obtained when a mixture of 23 amino acids (35) was substituted for yeast extract. By a process of elimination, we determined that both isoleucine and leucine were essential for growth (Fig. 2). Optimal growth rates and yields were obtained with 0.75 mM isoleucine and leucine (Fig. 3). The branched-chain fatty acids isovalerate and 2-methylbutyrate substituted for leucine and isoleucine, respectively. However, fatty acid concentrations of more than 4.4 mM were required for optimal growth (Fig. 4). The growth obtained with 2-methylbutyrate in the absence of leucine (Fig. 4) was probably due to contamination by isovalerate. Thus, in the presence of isoleucine a 10-fold-greater concentration of 2-methylbutyrate was required to produce growth equivalent to growth with isovalerate. This result was not surprising considering the difficulty in separating these isomers (12). Valerate, isobutyrate, and 5-methylcaproate did not substitute for either isoleucine or leucine.

Acetate requirement. In defined medium, acetate was required for growth (Fig. 5). Optimal growth occurred with 2.5 mM acetate. No

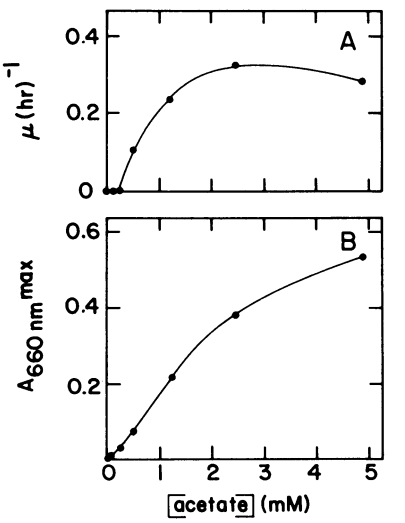

FIG. 5. Acetate requirement for growth. (A) Effect of acetate on the growth rate (μ). (B) Effect of acetate on the final absorbance at 660 nm (A_{660nm}).

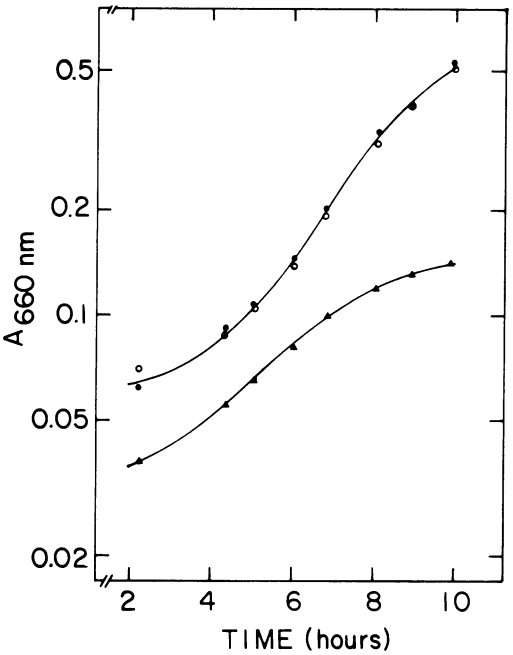

FIG. 6. Pantothenate stimulation of growth. Bacteria were grown in defined medium supplemented with 10 water-soluble vitamins (○) or 5 mg of pantothenate per liter (●), or they were grown in medium without vitamins (▲). The growth temperature was 30°C. The inoculum was grown in defined medium without vitamins. A_{660nm}, Absorbance at 660 nm.

growth was observed within 14 days with the following compounds (at concentrations of 2 g/liter, except where noted) in place of acetate: glucose, fructose, pyruvate, citrate, malate, citrate plus malate, aspartate, alanine, aspartate plus alanine, glutamate, glutamate plus aspartate, malate plus pyruvate, formate (0.1 g/liter), propionate (0.1 g/liter), butyrate (0.1 g/liter), valerate (0.1 g/liter), succinate (0.1 g/liter), and methylamine (0.1 g/liter).

Sulfur and nitrogen sources. Sulfide was required for good growth in defined medium. No difference in the growth rate or yield was found in the concentration range from 4.5 to 45 mM. However, a small amount of growth was observed in the absence of sulfide when cysteine was added before the medium was autoclaved. If cysteine was filter sterilized and added after autoclaving, no growth was observed. Therefore, a breakdown product of cysteine may also serve as a sulfur source. Cysteine, dithiothreitol, or sulfate (in the presence of cysteine) did not support growth in the absence of sulfide and did not increase either the growth rate or the final yield in the presence of sulfide.

Ammonia was required for growth of *M. voltae* in defined medium. Optimal growth was obtained at a concentration of 5 mM. No growth was observed within 14 days when other nitrogen-containing compounds were substituted for ammonia. The following compounds were tested (at concentrations of 5 mM, except where noted): nitrate, nitrite, methylamine, trimethylamine, taurine, urea, allantoic acid, glucosamine, 2-aminopurine, cytosine (0.2 mM), thymine (0.2 mM), uracil (0.2 mM), p-aminobenzoic acid (0.2 mM), isoleucine, leucine, glutamate, ornithine, cysteine, lysine (2 mM), histidine (2 mM), arginine (2 mM), aspartate (2 mM), alanine (2 mM), glycine (2 mM), asparagine (2 mM), and proline (2 mM).

Pantothenate requirement. *M. voltae* required none of the 10 vitamins added to the complex medium for growth. However, the addition of 5 mg of pantothenate per liter increased both the growth rate and the final yield in defined medium (Fig. 6). To determine whether pantothenate was stimulatory or was required in unusually low concentrations, all glassware was acid cleaned and heat treated (5). Medium prepared under these conditions was sufficiently free of contamination by vitamins to demonstrate a growth requirement for seven vitamins, including pantothenate, in a *Streptococcus* isolate (John Leigh, personal communication) and a growth requirement for 2-mercaptoethanesulfonate in *Methanobrevibacter ruminantium* strain M-1 (5). Under these conditions, growth was observed in the absence of pantothenate after six transfers (representing a final dilution of 10^9).

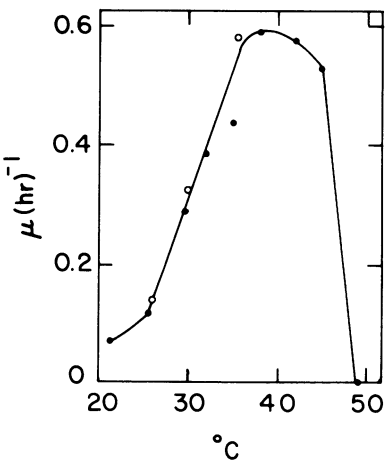

FIG. 7. Temperature optimum for growth. The bacteria were grown in complex medium. The inoculum at each temperature was from a culture grown at the next lowest temperature, except for the lowest temperature tested in each experiment. In this case, the inocula were grown at 24°C (●) and 30°C (○). The results of two separate experiments are shown. μ, Growth rate.

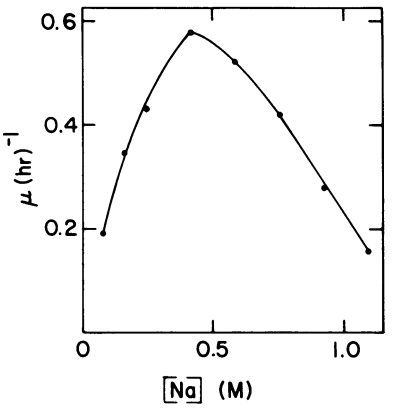

FIG. 8. Optimal NaCl concentration for growth. Bacteria were grown in complex medium containing 0.2% yeast extract and 0.2% Trypticase instead of Casamino Acids. This medium contained 75 mM sodium salts other than NaCl. Additional Na^+ was added as NaCl. The results are the growth rates (μ) obtained after three transfers in medium at each concentration of NaCl.

Therefore, pantothenate appeared to be stimulatory and was not absolutely required for growth, and no other vitamins were required for growth.

Substrates for methanogenesis. The growth rates with H_2-CO_2 (80:20) and formate (4 g/liter) as the substrates for methanogenesis were compared. In defined media between pH 6.0 and 7.0, the growth rates were 0.43 and 0.39 h^{-1} for H_2-CO_2 and formate, respectively. Acetate, methanol, and methylamine did not support growth.

pH and temperature optima. The initial pH of the medium was adjusted between 5.0 and 10.0 with bicarbonate, succinate, Tris, and other buffers, as described above. In all cases, no growth occurred below pH 5.8 or at 8.0 and above. The growth rate was optimal between pH 6.0 and 7.0. During growth on medium that initially was below pH 7.0, the pH of the medium rose to between 6.9 and 7.4 by the stationary phase. The temperature optimum for growth was between 35 and 45°C (Fig. 7). No growth occurred at 49°C. Furthermore, at 37°C rapid cell lysis followed stationary phase. After growth at 30°C, cell lysis was much less rapid.

Mineral requirements. In complex medium, *M. voltae* grew in a wide range of NaCl concentrations (Fig. 8). The optimal concentration was about 0.4 M, or very close to the concentration in seawater. However, the bacterium grew at one-third the optimal rate both in the absence of NaCl (but with 0.075 M Na^+) and in the presence of 1.03 M NaCl. At 0.4 M NaCl, increasing the concentrations of KCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, $Fe(NH_4)_2(SO_4)_2$, and trace minerals 10-fold had no significant effect on the growth rate.

To determine the metal requirements of *M. voltae*, Chelex-treated defined medium was prepared as described above. This medium was then supplemented with all of the metals and other inorganic compounds tested except the one for which a requirement was being sought. After repeated transfer, *M. voltae* failed to grow in medium lacking Fe, Ca, Ni, or Mg. After four transfers in the absence of Co, the growth rate was reduced from 0.17 h^{-1} in the presence of 2 μM $CoCl_2$ to 0.086 h^{-1} at 30°C. Furthermore, the addition of some rare metals (2 μM $PtCl_2$, 2 μM $PdCl_2$, 4 μM $IrCl_3$, 4 μM $RhCl_3$, and 15 μM $RuCl_3$) neither inhibited growth nor substituted for the Fe or Ni requirement. Titration of the required metals indicated that the maximum absorbance was limited by less than 1,000 μM $CaCl_2$, 10 μM Fe_2O_3, or 0.2 μM $NiCl_2$ (Fig. 9). In the case of Mg, the final absorbance was unchanged during growth with 84 to 0.084 mM $MgSO_4$, whereas the growth rate nearly doubled over the same concentration range (Fig. 10).

To determine whether additional metals were taken up by the bacterium, cells grown in either defined medium or Chelex-treated defined medium supplemented with the four required metals, 2 μM $CoCl_2$, and 10 μM Se_2O_3 were washed and analyzed by neutron activation (Table 1). Two metals, Zn and Mn, were accumulated by cells in defined medium, and a nutritional requirement for these metals could not be demonstrated. During growth on Chelex-treated medium, the amounts of these metals associated with whole cells were reduced greatly (Table 1). Nevertheless, sufficient Zn was still associated with whole cells so that a physiological role for this metal could not be eliminated. Indeed, Zn is required for activity of the NADPH-F_{420} reductase of *M. vannielii* (S. Yamazaki and L. Tsai, Fed. Proc. **39**:1698, 1980). In contrast, the con-

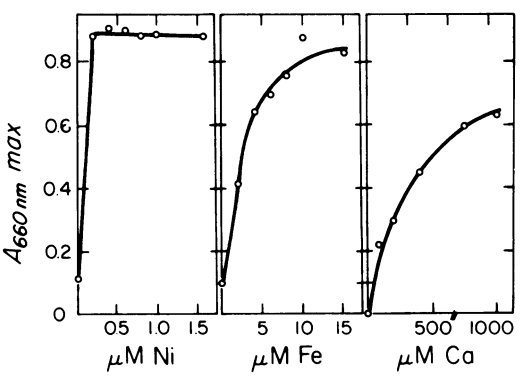

FIG. 9. Metal requirements for growth. The absorbance at 660 nm (A_{660nm}) at the end of growth was determined in Chelex-treated medium supplemented with the indicated concentrations of metals, as described in the text.

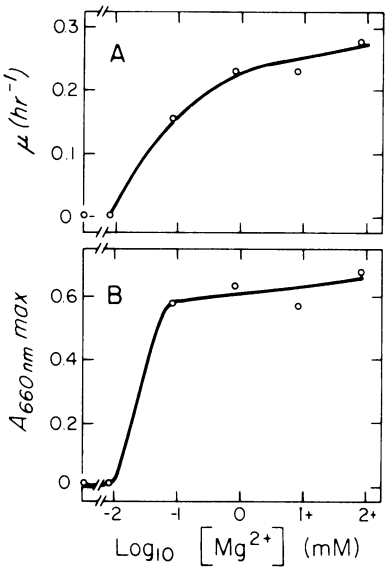

FIG. 10. Magnesium requirement for growth. (A) Effect of magnesium concentration on the growth rate (μ). (B) Effect of magnesium concentration on the final absorbance at 660 nm (A_{660nm}).

centrations of Mn associated with whole cells after growth on Chelex-treated medium were reduced to almost negligible levels. The reduction in the amount of K after growth in Chelex-treated medium may have been due to the more extensive washing procedure used (32). The levels of divalent cations are usually less sensitive to the washing procedure than the levels of monovalent cations (15).

Incorporation of ^{14}C-labeled substrates. We prepared cultures in which each of the four possible carbon sources was radiolabeled. As Table 2 shows, 46% of the total cellular carbon was derived from isoleucine and leucine. When cells were grown in the presence of [*U*-^{14}C]glutamate, an amino acid not required for growth, only 0.4% of the cellular carbon was labeled. Therefore, the high level of incorporation of isoleucine and leucine appeared to be specific for these amino acids. The remainder of the cellular carbon was obtained from the carboxyl (14%) and methyl (17%) carbons of acetate and from carbon dioxide (23%) (Table 2). Furthermore, 47.2% of the cellular dry weight could be accounted for as carbon; this was very close to the 50% determined for *Escherichia coli* (26).

The radiolabeled cells were fractionated as described by Roberts et al. (26). A total of 82% of the radiolabel was found in the alcohol-soluble, ether-insoluble and residue or protein fractions (Table 3). An additional 10.7% of the label was found in the hot TCA-soluble or nucleic acid fraction. The remainder of the label was found in the alcohol-soluble, ether-soluble or lipid fraction (2.6%) and the cold TCA-soluble fraction (4.8%). Because such a large percentage of the cellular carbon was found in the protein fraction, the residue was analyzed for protein and compared with the total radiolabel in that fraction. As determined by this method, 52.8 mg of carbon was labeled per 100 mg of protein. This ratio was close to the expected value of 51 mg of carbon per 100 mg of protein (26) and verified the proteinaceous nature of this fraction. The cellular composition of *M. voltae* appeared to be similar to that of *Methanospirillum hungatei*, another methanogen with a protein cell wall. Dry whole cells of *M. hungatei* are 71 to 81% protein and 13 to 18% nucleic acid, as determined chemically (8).

The distribution of radiolabel within the cellular components varied greatly with the labeled substrate (Table 3). Isoleucine and leucine carbon was incorporated almost exclusively into protein, accounting for 55% of the total label found in these fractions and only 7% of the label found in either the nucleic acid or lipid fraction. By contrast, acetate carbon was incorporated into all cellular components. The lipid fraction was particularly enriched in carbon from acetate. The carbon of carbon dioxide was incorpo-

TABLE 1. Metal content of whole cells of *M. voltae*

Metal	Concn (μg/g of dry wt) on:	
	Defined medium[a]	Chelex-treated defined medium[b]
Co	40 ± 1	64 ± 1
Fe	3,160 ± 400	990 ± 30
K	15,700 ± 500	11,500 ± 300
Mn	27 ± 1	1.7 ± 0.1
Na	3,610 ± 50	3,490 ± 50
Ni	<290	30 ± 7
Zn	1,810 ± 70	23 ± 3

[a] Cells were grown in defined medium, washed once with 0.2 M sucrose, and dried over P_2O_5 in vacuo. A total of 17 mg (dry weight) of cells was used for neutron activation analysis. In addition to the elements listed, we found significant amounts of the following elements (in micrograms per gram of dry weight): Sc, 0.656 ± 0.039; As, 6.8 ± 1.4; Se, 13.7 ± 3.8; Br, 6.6 ± 1.2; Sb, 1.34 ± 0.17; and Au, 0.21 ± 0.04.

[b] Cells were grown in Chelex-treated medium supplemented with 84 mM $MgSO_4$, 1 mM $CaCl_2$, 10 μM Fe_2O_3, 0.2 μM $NiCl_2$, 2 μM $CoCl_2$, and 10 μM Se_2O_3. After harvesting, the cells were washed four times with 0.2 M sucrose and dried over P_2O_5 in vacuo. A total of 82 mg (dry weight) of cells was used for neutron activation analysis. In addition to the elements listed, we found significant amounts of the following elements (in micrograms per gram of dry weight): Sc, 0.0753 ± 0.0036; Se, 320 ± 8; Br, 0.38 ± 0.05; Sb, 0.157 ± 0.010; Cs, 0.18 ± 0.05; Au, 0.017 ± 0.03; and Hg, 11.5 ± 0.26.

TABLE 2. Incorporation of ^{14}C-labeled compounds into whole cells of *M. voltae*[a]

^{14}C-labeled compound	^{14}C concn (mg/100 mg of cell dry wt)	
	Expt 1[b]	Expt 2[c]
[1-^{14}C]acetate	6.8	8.9
[2-^{14}C]acetate	7.8	9.1
$^{14}CO_2$	10.7	14.3
[*U*-^{14}C]leucine	11.7	
[*U*-^{14}C]isoleucine	10.2	
[1-^{14}C]isovalerate		2.2[d]
2-[1-^{14}C]methylbutyrate		2.5[d]

[a] Based on the radioactivity found in washed whole cells grown in defined media, as described in the text.

[b] In experiment 1, five cultures were grown in defined medium containing acetate, CO_2, and amino acids, as described in the text. One carbon source was radiolabeled in each culture, as indicated.

[c] In experiment 2, five cultures were grown in defined medium containing acetate, CO_2, and fatty acids, as described in the text. One carbon source was radiolabeled in each culture, as indicated.

[d] Calculated on the assumption that 60 mg of carbon was incorporated per mmol of ^{14}C incorporated.

rated largely into the protein and nucleic acid fractions. The ratio of the amount of total radiolabel incorporated from acetate to the amount of radiolabel incorporated from carbon dioxide was close to one in these fractions. In contrast, in the lipid fraction this ratio was close to 20. Thus, the carbon from carbon dioxide was not a major component of these lipids.

When [1-^{14}C]isovalerate and 2-[1-^{14}C]methylbutyrate were substituted for leucine and isoleucine, the total radiolabel incorporated was much less than the radiolabel incorporated from the amino acids (Table 2). Nevertheless, the incorporation of label from acetate and carbon dioxide was substantially unchanged when the cells were grown with the fatty acids rather than leucine and isoleucine (Table 2). Thus, the total amount of cell carbon radiolabeled by all substrates was reduced. Valerate, a fatty acid not required for growth, was not incorporated into cell carbon with the same efficiency as isovalerate and 2-methylbutyrate. During growth with 4.5 mM [1-^{14}C]valerate, no more than 0.4% of the cell carbon was labeled, assuming that all of the carbons of valerate were incorporated with the label. Therefore, the assimilation of isovalerate and 2-methylbutyrate appeared to be specific.

The distribution of radiolabel from [1-C^{14}]isovalerate and 2-[1-C^{14}]methylbutyrate among the cellular components was similar to that found for isoleucine and leucine (Table 4). The protein fractions contained 81% of the label. A small portion (3%) of the radiolabel was incorporated into lipids. Somewhat more (12%) of the radiolabel was found in the nucleic acid fraction. Assuming that all five carbons from isovalerate and 2-methylbutyrate were incorporated with the radiolabel in the lipid and nucleic acid fractions, no more than 7% of the total carbon in these fractions was derived from these fatty acids. Furthermore, if significant amounts of unlabeled carbon formed during the breakdown of the 1-^{14}C-fatty acids were incorporated into either the lipid or nucleic acid fraction, the amount of carbon incorporated from carbon dioxide and acetate should have been less than during growth on the amino acids. In fact, the opposite was observed (Table 4). Therefore, it is unlikely that the fatty acids provided a significant source of carbon for the biosynthesis of these cellular components. In contrast, fourfold less protein carbon was labeled by the 1-^{14}C-fatty acids than by the *U*-^{14}C-amino acids. Because there was no corresponding increase in carbon incorporation from carbon dioxide and acetate into protein in the presence of the fatty acids, this difference must have been due to the breakdown of the fatty acids and the incorporation of the unlabeled portions of the molecules. This observation also eliminated the possibility that the label from the fatty acids was diluted by exchange reactions with carbon dioxide.

TABLE 3. Distribution of ^{14}C-labeled carbon sources in whole cells grown with amino acids[a]

Cellular fraction	Amt of ^{14}C incorporated (mg/100 mg of cellular C) with the following ^{14}C-labeled carbon sources:[b]				
	[1-^{14}C]acetate	[2-^{14}C]acetate	[*U*-^{14}C]isoleucine	[*U*-^{14}C]leucine	$^{14}CO_2$
Cold TCA-soluble	1.1	1.2	0.1	0.1	2.3
Alcohol-soluble, ether-insoluble	0.7	0.7	0.8	1.3	1.1
Alcohol-soluble, ether-soluble	0.7	1.6	0.1	0.1	0.1
Hot TCA-soluble	2.7	2.4	0.4	0.4	4.8
Residue	9.1	10.6	20.3	23.0	14.3

[a] Cells were grown as described in Table 2, footnote *b*, in defined medium supplemented with isoleucine and leucine. Each of the carbon sources was labeled as indicated.

[b] Calculated as: (radioactivity incorporated per fraction) × (specific activity of the substrate)$^{-1}$ × (grams of carbon per mole of substrate) × (100 mg) × (0.427)$^{-1}$ × (gram [dry weight] of cells)$^{-1}$.

DISCUSSION

Several aspects of the nutrition of *M. voltae* and *M. vannielii* are strikingly different. *M. voltae* absolutely requires branched-chain amino acids or fatty acids and acetate for cellular carbon synthesis, but *M. vannielii* grows well in an essentially autotrophic medium (18), although it may be stimulated by two- and three-carbon intermediates (17). In addition, the growth of *M. voltae* is stimulated by pantothenate. No vitamin requirements have been reported for *M. vannielii*. The mineral requirements of these two bacteria also differ. *M. voltae* grows best with sodium and magnesium in concentrations comparable to those in seawater (about 0.4 M sodium and 80 mM magnesium). In contrast, *M. vannielii* does not grow in the presence of 0.5 M NaCl (17). The optimal concentration of magnesium for *M. vannielii* has not been determined. However, good growth is obtained with 1 mM magnesium (18). Although both bacteria grow well at a neutral pH with H_2 as the electron donor, *M. vannielii* requires a more alkaline pH than *M. voltae* for growth on formate (17). The inability of *M. voltae* to grow at pH 8 or greater may be a result of the requirement for high concentrations of magnesium and other divalent cations, which precipitate at high pH values.

Despite these differences, there are some important similarities in the nutrition of these bacteria. In contrast to an earlier report (4), the growth of both bacteria is stimulated by selenium (18). A newly isolated member of the genus, *Methanococcus maripaludis*, also requires selenium for optimal growth (W. J. Jones and M. J. B. Paynter, personal communication). A requirement for selenium has not been observed in other methanogenic bacteria. Thus, the selenium requirement may be a specific growth characteristic of the methanococci. Whether the apparent lack of a requirement for selenium by other methanogens is due to an inability to utilize this element or to a requirement for lower concentrations is not known. Selenium is probably present in most media as a contaminant of sodium sulfide.

As far as is known, all methanogenic bacteria can use ammonia and sulfide as their sole nitrogen and sulfur sources (23, 38). In addition, *M. voltae* does not use a wide range of organic compounds, including amino acids, purines, and pyrimidines, as sole nitrogen sources. This is surprising because *M. voltae* assimilates large quantities of leucine and isoleucine for cell carbon. Therefore, the inability to use amino acids as a sole nitrogen source cannot be due to a failure in transport. Likewise, members of the family *Methanobacteriaceae* restrict their nitrogen source to ammonia even in the presence of complex mixtures of amino acids and peptides (9). Of the limited number of sulfur compounds tested, sulfide was the only compound which supported growth of *M. voltae*. Cysteine did not support growth when care was taken to avoid autoclaving the compound. However, growth was observed when cysteine was either the sole sulfur or sole nitrogen source, provided that it was added to the medium before autoclaving. Sulfide and ammonia are formed readily upon heating cysteine in dilute base (11). Therefore, *M. voltae* probably utilizes the sulfide and ammonia formed from cysteine under these conditions.

Requirements for magnesium, calcium, iron, and nickel were demonstrated after treatment of the medium with Chelex-100, a strong chelator of divalent cations. However, several minerals of interest, such as molybdate and tungstate (18), are not removed by Chelex-100. Glass culture tubes and syringes were necessary to maintain strict anaerobiosis and may have been a source of contamination (30). Thus, a growth requirement for zinc was not demonstrated, but zinc was found associated with cells grown in Chelex-treated medium.

A requirement for calcium has not been described previously for methanogenic bacteria, although calcium does influence the degree of

TABLE 4. Distribution of ^{14}C-labeled carbon sources in whole cells grown with fatty acids[a]

Cellular fraction	Amt of ^{14}C incorporated (mg/100 mg of cellular C) with the following ^{14}C-labeled carbon sources:[b]				
	[1-^{14}C]acetate	[2-^{14}C]acetate	2-[1-^{14}C]methylbutyrate[c]	[1-^{14}C]isovalerate[c]	$^{14}CO_2$
Cold TCA-soluble	1.2	1.3	0.1	0.3	3.2
Alcohol-soluble, ether-insoluble	1.0	1.2	0.4	0.4	2.3
Alcohol-soluble, ether-soluble	1.1	1.8	0.1	0.2	0.3
Hot TCA-soluble	5.0	4.4	0.4	0.9	9.1
Residue	10.0	10.5	4.6	3.2	16.7

[a] Cells were grown as described in Table 2, footnote *c*, in defined medium containing 2-methylbutyrate and isovalerate. Each of the carbon sources was labeled as indicated.

[b] Calculated as described in Table 3, footnote *b*.

[c] Calculated on the assumption that 60 mg of C was incorporated per mmol of ^{14}C incorporated.

clumping of *Methanosarcina barkeri* (P. Scherer and H. Sahm, personal communication). Like the requirement for high concentrations of magnesium and sodium, the calcium requirement may reflect an adaptation to the marine environment. Nickel is also required for the growth of *Methanobacterium thermoautotrophicum* (29) and is a component of an unusual coenzyme, factor 430, which is found in methanogenic bacteria (14, 37). Factor 430 may also be present in *M. voltae*. Consistent with this idea is the fact that whole cells contain more than 10 times the amount of nickel found in marine eubacteria (15). Several species of *Methanobacterium* (24, 33) and *Methanospirillum* (24) require high levels of iron. The levels incorporated by whole cells of *M. voltae* are greater than the levels found in many aerobic eubacteria, but they are within the range found in bacteria in general (15). Cobalt is required for the growth of *M. thermoautotrophicum* (29). Likewise, cobalt stimulates growth and is incorporated into whole cells of *M. voltae*. The level of cobalt found in cells (40 to 64 μg/g) is comparable to the levels of corrinoids in other H_2-oxidizing methanogenic bacteria (0.65 to 0.91 nmol/mg of dry weight [21] or 38 to 54 μg of cobalt per g). Thus, the cobalt required by *M. voltae* may be used mainly for the synthesis of corrinoids.

Acetate is required for the growth of many methanogenic bacteria, including members of the *Methanobacteriaceae* (9) and *Methanospirillum* (25). Both *M. voltae* and *M. ruminantium* strain M-1 synthesize a large portion of their cellular carbon from acetate (9). The distribution of radiolabeled acetate into the cellular components is also similar. In the case of *M. ruminantium*, 60, 20, and 14% of the acetate carbon are found in proteins, nucleic acids, and lipids, respectively. In *M. voltae*, 69 to 61, 25 to 17, and 7.7 to 7.5% of the acetate carbon are found in these fractions, respectively. Like *M. voltae*, *M. ruminantium* requires volatile fatty acids (9). When *M. ruminantium* was grown with 2-[1-^{14}C]methylbutyrate, 93% of the radiolabel was found in the protein fraction of whole cells, and 4% of the radiolabel was found in the lipid fraction (27). The growth medium contained rumen fluid, which may have provided an alternative substrate for lipid biosynthesis. When *M. voltae* was grown in defined medium, the incorporation of label from the volatile fatty acids showed a silmilar pattern. For 2-[1-^{14}C]methylbutyrate, 89 and 2% of the label were found in the protein and lipid fractions, respectively. For [1-^{14}C]isovalerate, 72 and 4% of the label were found in these fractions, respectively. Thus, *M. ruminantium* and *M. voltae* appear to assimilate acetate and volatile fatty acids in similar fashions.

Isoprenoid lipids are the major lipids in *M. voltae* and other methanogens (34). In many rumen eubacteria, branched-chain lipids are synthesized from branched-chain volatile fatty acids (2, 36). Very little of the radiolabel from these sources was incorporated into the lipids of *M. voltae* and *M. ruminantium*. In *M. voltae*, more than 80% of the carbon found in the lipid fraction was derived from acetate. Therefore, the isoprenoid lipids may be derived from acetate via acetoacetyl coenzyme A (CoA) and not by the degradation of leucine. From the distribution of label from [^{13}C]acetate, De Rosa et al. (13) reached a similar conclusion for the biosynthesis of the isoprenoid lipids of another archaebacterium, *Caldariella acidophila*.

In rumen eubacteria, the volatile fatty acids are incorporated into protein after reductive carboxylation and amination (1, 3, 27). Thus, 2-methylbutyrate and isovalerate are used to synthesize isoleucine and leucine, respectively. Therefore, it is not surprising that *M. voltae* can use either the fatty acid or the amino acid, and both are incorporated largely into protein. Unlike the rumen eubacteria, *M. voltae* obtains 55% of its protein carbon and 47% of its cell carbon from these amino acids. The fatty acids are also metabolized and not simply incorporated intact. In contrast, during protein synthesis the rumen eubacteria incorporate the branched-chain fatty acids exclusively into branched-chain amino acids (27).

Other aspects of the utilization of leucine and isoleucine are unusual. Eubacteria oxidize leucine and isoleucine to acetoacetate and acetyl-CoA and propionate and acetyl-CoA, respectively. None of the intermediates in the synthesis of acetyl-CoA from leucine and isoleucine is common to the pathways for biosynthesis of other amino acids. Therefore, one-third of the radiolabel from isoleucine and leucine should appear as acetyl-CoA and dilute the exogenously derived acetate pool. Almost 31% of the cellular carbon is obtained from acetate. If one-third of the carbon from isoleucine and leucine appears in the acetate pool (15.5% of the total cellular carbon), the ratio between acetate carbon and carbon from isoleucine and leucine should be no greater than 2:1 in any cellular fraction. However, this ratio is 11.5:1 for the lipid fraction and 6.4:1 for the nucleic acid fraction. Thus, either acetyl-CoA is not a major product of isoleucine and leucine incorporation, or the acetyl-CoA formed is not in equilibrium with exogeneously derived acetate. Although the latter explanation may resolve part of the discrepancy, especially in the case of lipid biosynthesis on the cell membrane, it is difficult to envision it as the complete solution. It seems likely that *M. voltae* has a unique mechanism for

the conversion of isoleucine and leucine to other amino acids.

The distribution of radiolabel from acetate and carbon dioxide in nucleic acids is consistent with well-known biosynthetic pathways. If ribose (or deoxyribose) is synthesized by gluconeogenesis from acetate via the pentose phosphate pathway, 21% of the total nucleic acid carbon would be labeled by carbon dioxide and the C-1 of acetate. An additional 11% would be labeled by the C-2 of acetate. If pyrimidines are synthesized from carbamyl phosphate and aspartate and aspartate is synthesized from acetate and carbon dioxide via oxaloacetic acid, then 5% of the total nucleic acid carbon would be labeled by the C-1 and C-2 of acetate and 10% would be labeled by carbon dioxide. If purines are synthesized from glycine, carbon dioxide, and the C-1 units of folates (which in turn are derived from the C-2 of acetate via serine), then 10% of the total nucleic acid carbon would be labeled by carbon dioxide and the C-2 of acetate and 5% would be labeled by the C-1 of acetate. The sum of these pathways would be as follows: 41% of the total nucleic acid carbon would be labeled by carbon dioxide, 31% would be labeled by the C-1 of acetate, and 26% would be labeled by the C-2 of acetate. The actual distribution of the label found in the nucleic acid fraction was 45% from carbon dioxide, 25% from the C-1 of acetate, 22% from the C-2 of acetate, and 7% from isoleucine and leucine. Considering the uncertainty of the nature of the contribution by the amino acids to the labeling pattern, these results are in reasonable agreement with the expected values. This discussion merely demonstrates that a known pathway of nucleic acid biosynthesis is consistent with the observed labeling data. The actual pathway in *M. voltae* can be determined only after more detailed experiments.

The most recent taxonomy of methanogenic bacteria is based on phylogenetic relationships determined by sequence homologies of 16S rRNA (4). A comparison of the phylogeny with the nutritional characteristics of methanogenic bacteria demonstrates that nutrition has very little taxonomic significance for even closely related species. Thus, *M. voltae* is a salt-tolerant heterotroph, and *M. vannielli* is an autotroph which is inhibited by salt. Furthermore, distantly related organisms are nutritionally similar. The ability of *M. voltae* to utilize acetate and volatile fatty acids for cellular carbon is shared with *M. ruminantium*. Like *M. voltae*, *Methanogenium cariaci* and *Methanogenium marisnigri* are salt tolerant (28). Therefore, the nutritional characteristics must be determined largely by the environment and not the phylogeny of the organism. The nutritional diversity of closely related bacteria implies that there is a certain flexibility which enables adaptation to new environments. Thus, *M. voltae* may have lost the capacity for autotrophic growth as it gained the ability to utilize organic carbon. In contrast, *M. voltae* and *M. vannielii* have similar morphologies, cell wall structures, and lipid contents. These characteristics may be less sensitive to environmental effects than nutrition.

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