TU Delft
Bibliotheek
Prometheusplein 1
Postbus 98
2600 MG DELFT

Telefoon: 015 - 2784636
Fax: 015 - 2785673
Email: Helpdesk.doc@Library.TUDelft.NL

Uitsluitend voor eigen gebruik / for own use only

Datum: 10-mar-05

Bonnummer: 867389

Aan: PAQUES B.V
BIBLIOTHEEK
POSTBUS 52
8560 AB BALK
NEDERLAND

Tav:

Aantal kopieën: 15

Uw referentie(s): A077950216 mail 7-3-05

Artikelomschrijving bij aanvraagnummer: 867389

Artikel: Composition of the major elements and trace...
Auteur: Scherer, P and Lippert, HP
Tijdschrift: BIOLOGICAL TRACE ELEMENT RESEARCH
Jaar: 1983 **Vol.** 5 **Aflevering:**
Pagina(s): 149-165 **Plaatsnr.:** 4891

BIOLOGICAL TRACE ELEMENT RESEARCH 5, 149–163 (1983)

Composition of the Major Elements and Trace Elements of 10 Methanogenic Bacteria Determined by Inductively Coupled Plasma Emission Spectrometry

P. SCHERER*

Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg, Ohnhornststr. 18, D-2000 Hamburg 52, West Germany; Institut für Biotechnologie 1, Kernforschungsanlage Jülich, Postfach 1913, D-517 Jülich, West Germany

AND

H. LIPPERT AND G. WOLFF

Zentralabteilung für Chemische Analysen, Kernforschungsanlage Jülich GmbH, Postfach 1913, D-517 Jülich 1, West Germany

Received and accepted December 29, 1982

Abstract

The elemental composition of 10 methanogenic species was determined by inductively coupled plasma emission spectrometry and by a C-H-N-analyzer. The 10 species were representative of all three orders of the methanogens and were cultivated under defined conditions. Special emphasis was given to *Methanosarcina barkeri*, represented by 5 strains and cultivated on various substrates. The following elements with the lowest and highest values in parentheses were determined: C (37–44%, w/w), H (5.5–6.5%), N (9.5–12,8%); Na (0.3–4.0%), K (0.13–5.0%), S (0.56–1.2%), P (0.5–2.8%), Ca (order I: 85–550 ppm; order II: 1000–4500 ppm), Mg (0.09–0.53%), Fe (0.07–0.28%), Ni (65–180 ppm), Co (10–120 ppm), Mo (10–70 ppm), Zn (50–630 ppm), Cu (<10–160 ppm), Mn (<5–25 ppm). The biggest variations were found with respect to Na and K, which both seem to have important physiological functions. Although it is unknown whether zinc and copper are essential trace elements for methanogens, all investigated species contained remarkably high zinc contents, whereas copper seemed to be present only in some species.

©Copyright 1983 by The Humana Press Inc.
All rights of any nature whatsoever reserved.
0163–4984/83/0600–0149$03.00

Index Entries: Methanogenic bacteria, major and trace elements in; *Methanosarcina,* determination of trace and major elements in; sodium, in methanogenic bacteria; potassium, in methanogenic bacteria; magnesium, in methanogenic bacteria; iron, in methanogenic bacteria; cobalt, in methanogenic bacteria; zinc, in methanogenic bacteria; manganese, in methanogenic bacteria; sulfur, in methanogenic bactreia; phosphorus, in methanogenic bacteria; nickel, in methanogenic bacteria; molybdenum, in methanogenic bacteria; copper, in methanogenic bacteria; trace elements, determination in methanogenic bacteria; elements, determination in methanogenic bacteria; plasma emission spectroscopy, of elements in methanogenic bacteria; emission spectroscopy, of elements in methanogenic bacteria; spectroscopy, of elements in methanogenic bacteria; bacteria, determination of elements in; ICP, of methanogenic bacteria.

Introduction

Methanogenic bacteria are strict anaerobes at the end of anaerobic food chains, using H_2 plus CO_2 or formate as common carbon and energy source (Balch et al., 1979; Mah, 1981). However, there are some exceptions. For example, the species *Methanosarcina barkeri* is able to convert H_2 plus CO_2, H_2 plus CO, methanol, and acetate [Schnellen (*29*)] as well as methylamines [Hippe et al. (*1*)] to cell material and methane; it is therefore the metabolically most diverse, and perhaps the ecologically most relevant, methanogen. Together with the halobacteria and certain thermoacidophilic bacteria, the methaogens are considered to represent a line of early divergence from the eubacterial and eukaryotical lines of evolutionary descent and are designated "archaebacteria" [Woese (*38*)]. The elemental composition of eubacteria has not been analyzed extensively [for literature, see Jones et al. (*12*)]. In methanogenic bacteria, only a few elements were determined for the presence of one or two species [Sprott and Jarrell (*31*); Whitman et al. (*36*)]; and for four methanogens, only nickel was estimated [Diekert et al. (*3*)].

The intention of the present study was to confirm, through analyses, the known biochemical facts and the essential trace element requirements recently found for methanogens by growth experiments [Jones and Stadtman (*11*); Jarrell et al. (*10*); Scherer and Sahm (*27*); Schönheit et al. (*30*); and Whitman et al. (*36*)], especially those of Ni, Co, and Mo, and to estimate, at first in a representative number of specimens, the elements C, H, N, Na, K, S, P, Ca, Mg, Fe, Ni, Co, Mo, Zn, Cu, and Mn quantitatively.

Experimental

Organisms

All organisms with a DSM-No can be obtained from Dr. H. Hippe of the *D*eutsche *S*ammlung für *M*ikroorganismen (Göttingen, FRG), who also can supply all details of the isolations and references to the original literature. The organisms used are shown in Table 1.

TABLE 1
Sources of the Strains Used

Species	Designation of the strain	DSM-No	Donor source
Methanosarcina barkeri	'MS'	800	J. Winter, München/ Regensburg
Methanosarcina barkeri	'Fusaro'	804	DSM (H. Hippe)
Methanosarcina barkeri	'227'	1538	R. A. Mah, Los Angeles[b]
Methanosarcina barkeri	'Wiesmoor'	—	New isolate, P. S.[a]
Methanosarcina barleri	'Jülich'	—	New isolate, P. S.[a]
Methanosarcina spec. (50°C)	'TM-1'	1825	S. Zinder, Los Angeles/Ithaca (NY)
Methanosarcina vacuolata	—	1232	DSM (H. Hippe)
Methanococcus mazei	'S-6'	2053	R. A. Mah, Los Angeles[b]
Methanococcus vannielii	'SB'	1224	DSM (H. Hippe)
Methanobacterium bryantii	'M.o.H.G.'	862	DSM (H. Hippe)
Methanobrevibacter arboriphilus	'AZ'	744	DSM (H. Hippe)
Methanobrevibacter smithii	'PS'	861	G. Diekert, Marburg[c]
Methanobacterium thermoautotrophicum	'Marburg'	2133	R. K. Thauer, Marburg[d]
Methanobacterium spec.	—	—	New isolate, P.S.[a]

[a]New isolates, see text.
[b]This strain, which manifests itself as a sarcina or as a coccus morphotype (Mah and Smith, 1981) was three times grown as coccus.
[c]Cells were also cultivated by G. Diekert according to Balch et al. (*1*) with the help of yeast extract.
[d]Cells were cultivated with 50 μM $FeCl_2$ and 50 mM KH_2PO_4 at 65°C (Diekert et al., 1981).

New isolates: *Methanobacterium* spec. was isolated from a sewage digester using antibotics (Godsy, 4a) and continuously employing H_2CO_2 as the sole substrate. This bacterium might be identical with *Methanospirillum hungatei,* although the length is only about 5 μm [Balch et al. (*1*)]. It is not identical with a rodshaped *Methanobacterium* spec. isolated from a methanol consuming *Methanococcus* (manuscript in preparation). *M. barkeri* 'Jülich', a flocculating strain, was isolated via a microfloc from a sewage digester near Jülich. *M. barkeri* 'Wiesmoor', obtained by the Hungate technique as single colony from a roll tube (*1*), was isolated from a drainage creek of the peat bog 'Wiesmoor' (near Wilhelmshaven, FRG).

Cultivation and Culture Media

The pure cultures were cultivated under strict anaerobic conditions using a modified Hungate technique as described previously [Scherer and Sahm (*26*)]. An

optimized mineralic (*26*) medium was used, containing optimized concentrations of essential trace elements and the vitamins riboflavin and folic acid, as found earlier for *M. barkeri* (*27*). The combined basal medium contained, in deionized distilled (1×) water: imidazole-HCl buffer, 40 m*M* (adjusted by HCl to the given pH); NaH_2PO_4, 0.5 m*M;* Na_2HPO_4, 0.5 m*M;* $MgCl_2$, 2 m*M;* $CaCl_2$, 2 m*M;* KCl, 5 m*M;* NH_4Cl, 10 m*M;* NaCl, 34 m*M;*, $NiCl_2$, 5 μ*M;* $CoCl_2$, 1 μ*M;* $NaMoO_4$, 0.5 μ*M;* Na_2SeO_3, 0.1 μ*M;* riboflavin, 3 μ*M;* folic acid, 1 μ*M;* resazurin (colorless), 4.4 μ*M;* Ti-III-(citrate)$_2$ as made by $TiCl_3$, Na_3-citrate and NaOH (*26*), 0.18 m*M;* L-cysteine-HCl, 0.85 m*M*.

We used 0.5 L culture vessels (2.3 L total) because less contamination was caused by the stainless steel hypodermic syringe needles, when compared to 5 mL culture tubes with which the optimizations were done. The Na_2S content of the medium solutions was according to Scherer and Sahm (*26*) ≤50 μ*M* (S^{2-} + HS^-). To sustain such a concentration in the 0.5 L vessels at the specific pH (Table 2), Na_2S, to a final concentration of 0.1–0.3 m*M*, had to be added by syringe, after sterilization, in ¼ portions. The addition of Na_2S after sterilization reduces the quantities of Na_2S to be added drastically and avoids precipitations caused by sulfides (Na-phosphate, $NaHCO_3$, and Fe-II-solutions were also added after autoclaving). The growth under H_2 plus CO_2 (otherwise argon) was performed at 300 kPa and at 100 rpm on a rotary shaker. The temperature of growth was generally 37°C. Further conditions are summarized in Tables 1 and 2.

Analytical Procedures

The multielement analysis was done simultaneously for each sample with a multichannel emission spectrometry system with inductively coupled argon plasma (Bausch and Lomb, type 34000 ARL, Applied Res. Lab., Lausanne, Switzerland). Bacterial cells harvested in the early stationary growth phase were washed twice with water. This washing procedure was chosen because it is believed to generate the smallest bleeding artifacts in the cells with respect to the leaching of monovalent cations such as Na and K (*12*). Generally, two different cell batches cultivated with a different preculture were analyzed. For digestion, 1 mL conc. HNO_3 was added to lyophilized cell material (up to 50 mg), and this mixture was heated for 15 min at 150°C. After this, 0.5 mL conc. $HClO_4$ was added and the sample was heated to ca. 175°C, until white fumes appeared. The samples were subsequently diluted to a final volume of 10 mL, using quartz-tridistilled H_2O (to avoid interferences occurring at too high ionic strengths). The following conditions were employed at the instrument: flushing time, 10 s; flow rate, 2 mL/min (free running Meinhard nebulizer); integration time, 10 s; and number of integrations, 3. The plasma was adjusted to 1250 W at 27.15 MHz (about 10,000°C, thereby avoiding most of the molecular interferences). All elements were extensively standardized with standards in aqueous solutions. Analytical procedures were done with chemicals of "suprapure"-grade (Merck, Darmstadt, FRG) whereas cultivations in general were carried out with chemicals of analytical grade.

TABLE 2
Cultivation of Methanogenic Bacteria in Defined Media—Variations of the Basal Medium (see Experimental)

	Carbon source						Carbon source (supplem.)							
Organisms	H_2–CO_2 80:20	Methanol, 170–190 m*M*	Na-acetate, mM	Na-formate, m*M*	$NaHCO_3$, mM	$N(CH_3)_3 \cdot HCl$, m*M*	Na-citrate, 2 m*M*	Malate, 1 mM	L-Methionine, 1 m*M*	Vitamin solution (*39*)	Trace element solution, Hippe et al., 1979	Source of iron, μ*M*	$CaCl_2$, 0.1m*M* instead of 2 mM	pH (t_o)
DSM 862	+	–	5	5	10	–	–	+	–	+	+	50 $(NH_4)_2Fe(SO_4)_2$ 50 Fe II-citrate	+	6.8
DSM 744	+	–	5	5	10	–	–	+	–	+	+	50 Fe II-citrate	+	6.8
Mb. spec.	+	–	5	5	10	–	–	+	–	+	+	25 Fe II-citrate	+	6.6
DSM 1224	–	–	–	100	5[a]	–	–	–	–	+	+	50 $FeCl_2$[b]	–	8.0
DSM 800	–	+	–	–	5[a]	–	–	–	–	–	–	100 $(NH_4)_2Fe(SO_4)_2$ 100 Fe II-citrate	–	6.3
DSM 804	–	+	–	–	–	–	–	–	–	–	–	100 $(NH_4)_2Fe(SO_4)_2$ 100 Fe II-citrate	–	6.3
DSM 804	–	–	115	–	–	–	–	–	–	+	+	100 $(NH_4)_2Fe(SO_4)_2$	–	6.2
DSM 804	+	–	–	–	–	–	–	–	–	+	+	100 $Fe(SO_4)_2$	–	6.4
DSM 804	–	–	–	–	–	100	–	–	–	–	–	100 $(NH_4)_2Fe(SO_4)_2$ 100 Fe II-citrate	–	6.5
DSM 1232	–	+	–	–	5[a]	–	–	–	–	–	+	100 Fe II-citrate	–	6.3
DSM 1538	–	+	–	–	5[a]	–	–	–	–	+	+	100 $(NH_4)_2Fe(SO_4)_2$ 100 Fe II-citrate	–	6.3
DSM 1825	–	+	–	–	5[a]	–	+	–	+	+	+	100 $(NH_4)_2Fe(SO_4)_2$ 100 Fe II-citrate	–	6.4
DSM 2053	–	+	–	–	5[a]	–	–	–	–	–	+	50 $FeCl_2$[b]	–	6.3
Ms. barkeri Juelich	–	+	–	–	5[a]	–	–	–	–	–	+	50 $FeCl_2$[b]	–	6.3
Ms. barkeri Wiesmoor	–	+	–	–	5[a]	–	–	–	–	–	–	100 Fe II-citrate	–	6.3

[a]$NaHCO_3$ was used preventively and its necessity for good growth was not checked (*8*). [b]A 0.1*M* $FeCl_2$ solution was complexed by 0.1*M* Na_3-citrate and served as stock solution.

The C-H-N analysis of the same cell sample was separately performed with a Perkin-Elmer C-H-N analyzer (type 2400, Überlingen/Bodensee, FRG) as described in the apparatus manual.

Results

Methanogenic bacteria are regarded as fastidiously growing organisms and are often cultivated with the help of yeast extract because the nutritional requirements have been determined in only a few cases [for a review, see Mah and Smith (*18*) as well as Scherer and Sahm (*26, 27*), and Whitman et al. (*36*)]. For the present comparative study, the use of defined media was an advantageous prerequisite since it provides a reproducible nutritional starting base for all analyzed isolates. One part of the work was therefore the development of defined media allowing good growth of these pure cultures. Table 2 comprehends the elucidated growth conditions. However, for many additives, the direct optimum concentration was not worked out, and vitamins according to Wolin et al. (*39*) as well as a trace element solution [Pfenning and Lippert (*22*) as varied by Hippe et al. (*7*)] were often employed as a preventive measure without determining their essentiality.

Since methanogens are a special archaebacterial group (*38*) their C-H-N contents might well be different from those of eubacteria. Table 3 summarizes the results of the C-H-N analyses. With the exception of *M. arboriphilus* having a C content of only 37.1% (w/w), the values range from 40.3 to 44.1% carbon. In the case of *M. barkeri,* two probes grown under slightly modified conditions were analyzed. Their C-content varied as well, by nearly 10%, and the nitrogen content of the cells was directly correlated with the carbon content. The lowest N value was again found in *M. arboriphilus,* representing 9.5% of the analyzed cell material; the remaining values ranged from 10.3 to 12.8% of N. The resulting C : N ratios of 3.4–4.1 also manifest some variance.

Table 4 shows the results of the multielement analyses in descending order. It could be seen that the sodium content of the cells varies widely, from 0.3 to 4.0%, just as the K-content (0.13–5.4%). In the first five lines of Table 4, representing H_2–CO_2 specialists (Order I, Methanobacteriales), the Na contents were in general clearly lower than the corresponding K contents, whereas the formate and H_2–CO_2 utilizer *M. vannielii* (order II, Methanococcales) and the "methylotrophic" methanogens (order III, Methanomicrobiales) were very heterogeneous with regard to the elements Na and K.

Sulfur and phosphorus are major elements of all organisms, including those of the present study. The S content was approximately 0.6–1.0% in the methanogens growing normally on H_2–CO_2 or formate (order I and II) and in general somewhat higher in the "methylotrophic" methanogens (order III), revealing contents of 0.8–1.2% of sulfur. With respect to phosphorus, the relation is reversed. The P content present was 2.0–2.8% for orders I and II, being therefore generally higher, and 0.5–1.9% for the investigated species of order III.

Table 3
C-H-N Analyses of Methanogenic Bacteria[a]

Species	Strain	DSM-No	Substrate	Carbon	Hydrogen	Nitrogen	C:N
Methanobacterium bryantii	'M.o.H.G.'	862	H_2–CO_2	42.6 ± 0.2	10.3 ± 0.07	6.42 ± 0.05	4.1
Methanobacterium arboriphilus	'AZ'	744	H_2–CO_2	37.1 ± 0.3	9.5 ± 0.04	5.54 ± 0.002	3.9
Methanobacterium spec.	—	—	H_2–CO_2	40.3 ± 0.2	10.5 ± 0.1	6.27 ± 0.03	3.8
Methanobacterium barkeri	'227'	1538	MeOH	43.5 ± 0.3	11.5 ± 0.07	6.07 ± 0.03	3.8
Methanobacterium barkeri	'Fusaro'	804	H_2–CO_2	40.3 ± 0.2	11.1 ± 0.1	6.12 ± 0.02	3.6
Methanobacterium barkeri	'Fusaro'	804	MeOH	44.1 ± 0.1[b]	12.8 ± 0.07[b]	6.53 ± 0.03	3.4
Methanobacterium barkeri	'Fusaro'	804	MeOH	41.1 ± 0.1[c]	11.6 ± 0.02[c]	6.15 ± 0.02	3.5

[a]The values refer to the lyophilized cell material in percent (w/w).

[b]Cells were cultivated semicontinuously in a fermenter while stirring (100 rpm). The cell material was kept for several days in the stationary phase (without substrate at 4°C).

[c]Cells were cultivated as a batch culture (like the other ones; exceptions were DSM 2133 and 'Fusaro' grown on H_2–CO_2 in a fermenter) and harvested in the early stationary growth phase.

Table 4
Multielement Analyses of Methanogenic Bacteria as Revealed by Inductively-Coupled Plasma Emission Spectrometry[a]

Species	Strain/ DSM-No	Sub-strate	Na	K	S	P	Ca	Mg	Fe	Ni	Co	Mo	Zn	Mn	Cu
M. bryantii[b]	'M.o.H.G./ 862	H_2–CO_2	7000	32,500	5650	19,500	180	1900	1350	150	20	25	250	5	160
M. thermoauto-trophicum[b]	'Marburg' /2133	H_2–CO_2	5650	54,500	6350	26,000	85	890	1500	110	10	25	50	<5	<10
M. arboriphilus[d]	'A.Z.'/744	H_2–CO_2	3650	52,500	9900	28,500	550	3900	1300	65	15	40	440	5	20
M. smithii[c]	'PS' 861	H_2–CO_2	9000	50,000	7700	25,500	100	2250	1100	95	90	25	630	5	25
Methano-bacterium spec.[b]	—	H_2–CO_2	16,000	10,500	8300	24,500	(<950)	3900	2850	90	120	65	(>1000)	20	(<300)
M. vannielii[c]	'SB'/ 1224	Na-formate	27,000	7000	9200	25,500	(>>1000)	3650	(<5000)	70	85	60	(<1100)	25	30
M. mazei[c]	'S-6'/2053	Meth-anol	40,000	7700	7850	11,500	1700	2900	720	95	35	<10	170	5	10
M. vacuolata[b]	—/1232	Meth-anol	4450	1300	10,500	9500	4200	2800	1600	70	30	30	260	15	40
Methano sarcina spec.[b]	'TM1'/1825	Meth-anol	10,500	1400	12,000	5200	1800	2000	1600	150	50	<10	230	5	<10
M. barkeri[b]	'MS'/800	Meth-anol	4450	4900	12,000	11,500	4300	3100	1100	65	30	25	300	10	10
M. barkeri[b]	'227'/1538	Meth-anol	4550	5100	10,500	18,000	2750	5350	1200	60	25	45	370	10	35

M. barkeri[b]	'Wiesmoor'	Methanol	4350	9850	10,500	16,500	2250	2850	1530	180	80	70	55	5	<10
M. barkeri[b]	'Jülich'	Methanol	2950	8150	10,500	13,000	3600	4100	1100	60	30	20	150	5	<10
M. barkeri[b]	'Fusaro'/804	Methanol[f]	9200	2500	11,000	12,000	3800	1700	2150	135	60	60	130	5	10
		Methanol[g]	5200	17,000	11,000	17,000	3900	3950	1800	130	75	60	140	5	<10
		Methanol[g]	8200	10,000	8100	19,000	3800	4700	—	—	—	—	—	—	—
M. barkeri[b]	'Fusaro'	H_2–CO_2	13000	7750	8400	12,000	4500	4300	1400	70	—	35	(<670)	40	40
M. barkeri[c]	'Fusaro'	Na-acetate	(6850)	(5000)	(11,500)	(12,000)	(<5000)	(2850)	—	(180)	(85)	(40)	(<690)	(20)	(<100)
M. barkeri[e]	'Fusaro	$N(CH_3)_3 \cdot HCL$	—	6900	12,500	9800	2550	2200	1500	130	40	<50	(<780)	<20	60
		Detection limit (b):	3.2	7.5	8.0	5.7	0.006	0.016	0.6	0.6	0.34	0.34	0.2	0.06	0.24
		(c):	3.9	9.3	10.0	7.0	0.008	0.02	0.7	0.7	0.43	0.43	0.25	0.07	0.3
		(d):	8	18	20	14	0.015	0.04	1.5	1.5	0.9	0.9	0.5	0.14	0.6
		(e):	29	62	67	50	0.05	0.15	5	5	3	3	1.6	0.5	2

[a]Values are given in ppm of the lyophilized cell material (10,000 ppm = 1% w/w). Analytical error (relative): ±5% for Na, K, S, P, Ca, Mg; for the remaining elements: ±20% for values ~100 ppm and 50% for values <50 ppm. The values in parentheses are regarded as uncertain.

[b–e]The detection limits are given in ppm for aqueous standard solutions that have to be multiplied by a factor of 2–3 for the dissolved bacteria cells. The detection limits changed depending on the analyzed weight of cell material.

[f]Cells were cultivated semi-continuously in a fermenter while stirring (100 rpm). The cell material was kept for several days in the stationary phase (without substrate at 4°C).

[g]Cells cultivated as a batch culture (like the other ones; exceptions were DSM 2133 and 'Fusaro' grown on H_2CO_2 in a fermenter) and harvested in the early stationary growth phase. The cells of this batch cultures were analyzed for further elements (values in ppm): Al, 40 ± 14; Si, 35 ± 7; Cd, 2.5±1.5; Cr, 4 ± 1.5; Sr, 4 ± 0.8; Ba, 11 ± 3.

Calcium and magnesium are also elements of general importance. Table 4 shows that members of orders II and III had very remarkable Ca contents, lying between 0.18–0.45%. *M. barkeri* 'Fusaro' (order III), which was analyzed most intensively, revealed, in the case of methanol as carbon source, a conspicious uniformity of the Ca content between the different batches not seen in the previously presented elements. Furthermore, it was striking that the Ca content estimated for the H_2–CO_2 utilizers belonging to order I was a magnitude lower, ranging from only 85 to 550 ppm. The lowest value of 85 ppm was estimated for *M. thermoautotrophicum,* cultivated without the addition of Ca in the medium, thereby indicating that contaminating traces of Ca must have been present in this culture medium.

In comparison to calcium, magnesium was present in all methanogens at high levels. Values of 0.09–0.53% could be measured. The iron content of the methanogens was, with some exceptions, quite uniform, with an average value of 0.11–0.15%. In addition to the Fe values, Table 4 also shows the contents found for such "typical" trace elements as Ni, Co, Mo, Zn, Cu, and Mn, which were preventively added, for most of the examples, to the culture media (Table 2). Nickel was found to be present, usually at levels of 60–150 ppm in the cell material, with an exceptional high value of 180 ppm estimated for *M. barkeri* 'Wiesmoor'. This strain also had the highest molybdenum content of 70 ppm. On the other hand, Mo values were generally low, in the order of 25–60 ppm. Two species, *Methanosarcina* spec. 'TM-1' and *M. mazei* 'S-6', contained very low levels of <10 ppm Mo. The element cobalt was estimated to be present at somewhat varying levels. In some H_2–CO_2 utilizers (order I), values of 10–20 ppm were found. Other members of the same order, in contrast, contained 90 ppm or even 120 ppm of Co. *M. vannielii,* a representative of order II, offered 85 ppm Co and the Methanosarcinae (order III) contained 25–80 ppm Co.

Zinc values varied surprisingly. The levels varied conspicuously, with no recognizable pattern, although they were in general above 100 ppm. In *M. thermoautotrophicum* (order I) and *M. barkeri* 'Wiesmoor' (order III), levels of 50 and 55 ppm Zn, respectively, were estimated. The other species were found to contain 130–630 ppm Zn. The copper contents also showed wide variations. Several species contained 10 ppm Cu, e.g., *M. thermoautotrophicum* (grown on H_2–CO_2), *Methanosarcina* spec. 'TM-1' and *M. barkeri* strain 'Wiesmoor', 'Jülich,' or 'Fusaro' (grown on methanol). The remaining species contained from 10 to 40 ppm Cu. However, in some instances, e.g., in the case of *M. barkeri* 'Fusaro' grown on trimethylamine, a Cu content of 60 ppm was observed, and *M. bryantii,* grown on H_2–CO_2, even contained as much as 160 ppm. It is worth noting that the strain 'Fusaro' was generally cultivated without the addition of a trace element solution (Table 2) and therefore without the addition of copper. But the addition of the trace element solution to the medium of strain 'Fusion' (methanol as substrate) apparently did not alter the contents of Cu, nor of Ni, Co, Mo, and Zn (data not shown) significantly. Very low contents were estimated for manganese, mainly <5 ppm or 5–10 ppm. The highest value found was 25 ppm for *M. vannielii.*

Discussion

Before the results are discussed, some principal sources of error shall be considered; these arise mainly from the organisms themselves. Like many other anaerobes, the methanogenic bacteria require phosphate, carbonate, and sulfide for growth (*1, 8, 18, 26*). These anions can cause precipitations of $CaPO_4$, $CaCO_3$, and FeS. An additional source of error could come from adhesive hydroxides, mainly at pH values above 7, especially in the case of *M. vannielii* grown on formate and of *M. barkeri* grown on acetate because after consumption of these substrates, the pH values of the culture media become alkaline. To reduce such problems, special care was taken with medium preparation, and in addition, unusually low start- and growth-pH values were chosen for the cultivation.

The most important errors generally arise when washing the cells, as was pointed out by several authors [Takacs et al. (*34*); Kung et al. (*15*); Jones et al. (*12*)]. This especially concerns the monovalent cations of sodium and potassium, the measured values of which are often too low. Lysis or leakage of bacterial cells during the washing procedure or during growth could also be caused by cell damage owing to a lack of nutrients (this needs not to be apparent) or by the growth phase with respect to the age of the cells [Williams (*37*); Kung et al. (*15*); Sprott and Jarrell (*31*)]. Thus, the list of cation contents of eubacteria given by Bowen (*2*) should be used with caution: Na, 4600 ppm; K, 115,000 ppm; S, 5300 ppm; P, 30,000 ppm; Ca, 5100 ppm; Mg, 7000 ppm; Fe, 250 ppm; and Mn, 30 ppm. To diminish the described interferences, the analysis was performed with cells of nearly the same growth phase. The C-H-N contents of bacteria can also vary during the growth phase [Herbert (*6*)] or even the generation time. Esener et al. (*4*) reported for eubacterium *Klebsiella pneumoniae* that, during growth in a chemostat, decreasing generation times effected a decrease of C (mean value, 53%) and of H (mean value, 7%) and a weak increase of N (mean value, 13%) with maximum deviations of approximately 5%. These values may also serve as an example of the eubacterial group. Literature on the C-H-N content of a methanogen was not found. Recently, the N content of one thermophilic methanogen was determined to be 8.4%, and the ratios of C:N for several methanogens were estimated to be 4.5–4.9 [Rönnow and Gunnarson (*23*)].

Whereas the requirement of potassium for prokaryotes is well established (*37*), the need of sodium for the growth of prokaryotes is expressed only by marine bacteria, halophilic bacteria, and certain rumen bacteria [see Perski et al. (*21*)]. The relative importance of potassium in contrast to sodium might reflect also the list of cations given by Bowen (*2*). But for some microorganisms, e.g., *Escherichia coli*, it is well known that the transport of nutrients such as glutamate is mediated by symport with sodium [Lanyi (*16*)]. The very high values estimated here for Na (up to 4%) and for K (up to 5%) strongly suggest that potassium, but also sodium, should have an important physiological function in methanogens. In conformity with this, a proposed hypothesis considers a cation–proton antiport system acting in methanogens to explain a proton motive force in the absence of the transmembrane ΔpH [Jarrell and Sprott (*9*)].

The importance of K and Na was also validated by growth experiments. Recently, it was found that the growth and methane production of *M. thermoautotrophicum* was highly stimulated by sodium (the apparent K_m for Na^+ is approximately 1 m*M*). This was true also for resting cells for which a concentration of 50 m*M* Na^+ in the incubation medium was recommended (*21*). With resting cells of *M. barkeri* in a sodium-rich medium, conversion rates that increased by one order of magnitude were observed [Scherer et al. (*28*)]. Sodium dependence of *M. barkeri* was studied in more detail by Perski et al. (21a). In agreement with this is the optimized medium of *M. barkeri* containing about 35 m*M* NaCl (0.09%) and 5 m*M* KCl (0.02%) as previously described [Scherer and Sahm, 1979, (*26*)] and employed here for methanogens in general. However, somewhat contradictory results were obtained from Patel and Roth (*19*) and from Sprott and Jarrell (*31*). These authors did not observe similar Na-dependent effects. Sprott and Jarrell also estimated the amounts of Na, K, and Mg in *M. hungatei* and *M. thermoautotrophicum* as internal millimolar concentrations and with respect to three growth phases. Although no conversion factors for ppm were given, their values seem to be mostly lower than the values presented here (an exception was the high K content of *M. thermoautotrophicum*). In addition, Whitman et al. (*36*) reported for *Methanococcus voltae* (closely related to *M. vannielii*, order III) elemental values estimated by means of two different batches (the medium and the used H_2O was changed) 0.35/0.36% Na and 1.15/1.57% K as well as 990/3160 ppm Fe, 30/290 ppm Ni, 64/40 ppm Co, 23/1810 ppm Zn, 320/13.7 ppm Se, and 1.7/27 ppm Mn.

Recently, it was stated that a requirement of Ca, Mn, Cu, Zn, Al, and B for the growth of *M. thermoautotrophicum* (order I) could not be demonstrated (*30*), whereas *M. barkeri* 'Fusaro' (order III) needed high Ca concentrations for optimal growth [Scherer and Sahm, (*26*)] leading in its absence to macroscopic visible flocs of the cells. Another methanogen of order II also required Ca (1 m*M*) for optimal growth (*36*). From Table 4, it is striking that all Methanobacteriales (order I) contained more than a magnitude lower Ca contents than members of order II or III, thereby supporting the validity of the growth experiments. The results furthermore show that there seems to exist no correlation between growth substrate and this unusual Ca requirement. There could have been a correlation caused by differences in the composition of the cell wall; the envelope of *M. vannielii* (order II) is probably composed of protein subunits, while *M. barkeri* (order III) is enveloped in a different manner by heteropolysaccharides [Kandler and König (*13*)].

Iron, primarily because of its redox properties, is an essential element for all anaerobes. Although the additions of ferrous salts to the growth media were varied from 25 to 200 μ*M* [*M. smithii* grown even with 7.2 μ*M* $FeCl_2$, according to Balch et al. (*1*)], this resulted only in a relatively uniform content of approximately 1100–1500 ppm Fe, indicating that the concentration of 25 μ*M* of Fe-II was sufficient for 0.9 g cells/L (Table 2). Whitman et al. (*36*) optimized the "Fe_2O_3" concentration for *M. voltae* to 10–15 μM, but Patel et al. (*20*) in contrast derived optimal concentrations of 0.3–0.9 m*M* Fe, for the growth of several methanogenic pure cultures.

Perhaps nickel and cobalt could represent the most typical markers for methanogenic bacteria because they may function during catabolism. Methanogens ("methylotrophes" and H_2–CO_2 users, too) contain an unusual Ni-tetrapyrrol acting in the last step of methane generation [Diekert et al. (*3*); Vogels et al. (*35*)], a Ni-containing hydrogenase [Graf and Thauer (*5*)] and 5-hydroxylbenzimidazolylcobamide (factor III) [Stadtman and Blaylock (*32*); Scherer and Sahm (*25, 27*)]. Our analysis shows, in general, higher values for Ni [see also Diekert et al. (*3*)] than those of Co, which again were higher than the Mo contents. The results are in good agreement with the growth experiments involving Ni, Co, and Mo [Jarrell et al. (*10*); Scherer and Sahm (*27*); Schönheit et al. (*30*); and Whitman et al. (*36*)]. The physiological function of Mo in methanogens is not known, but an isolated Mo-containing CO_2 reductase of the anaerobe *Clostridium pasteurianum* gives a hint that Mo could be a possible candidate involved in the steps of CO_2 fixation [Scherer and Thauer (*24*)].

Cobalt, in combination with manganese (but not manganese alone), was reported to be stimulatory for growth of *M. barkeri* '227' on methanol [Mah et al. (*17*)]. Analogous to this, a dependence of growth on $MnCl_2$, $CuCl_2$, and H_3BO_3 could not be recognized for *M. barkeri* 'Fusaro' (*27*), and the same was also given for *M. thermoautotrophicum* (*30*). Our values of Mn were the lowest and suggest that it has only minor importance for methanogens. Also, biochemical data do not exist for copper, but in the instance of *M. bryantii*, the high value of 160 ppm found for Cu should be an indication for its essential, but thus far unknown, role in this bacterium.

Zinc can be regarded as an superacid catalyst (*37*) that functions in many enzymatic processes [Suelter (*33*)]. Remarkably high were the Zn levels determined for *all* methanogens, ranging from 50 to 630 ppm, but positive effects in growth experiments were not reported so far. The water used to cultivate the methanogens (not that for the analysis), came from a distilling apparatus having iron rods coated with zinc as the heating elements (type EWS-15, Schott, Mainz, FRG) and, in addition, the tap water is often contaminated by zinc-coated water supplies. Whitman et al. (*36*) also found a high zinc content for *M. voltae* (23–1800 ppm), but they could not determine a requirement of Zn for growth. For *M. barkeri* it was shown that ZnS or FeS (instead of Na_2S) could serve as an alternative sulfur source (*26*). Although *M. bryantii* possesses a superoxide dismutase containing 2.7 Fe and 1.7 Zn per tetramer, Zn was not considered to be an essential component [Kirby et al. (*14*)]. It is also unclear whether the NADPH-F_{420} oxidoreductase of *M. vannielii* 'SB' is a Zn-containing enzyme [Yamazaki and Tsai (*40, 41*)].

The high contents of Zn, Cu, Na, and K should stimulate the characterization of methanogenic archaebacteria with regard to these elements much more in detail.

An effort was also made to determine the selenium content of the different species by the same analytical procedure. But because the sensitivity of emission spectroscopy for selenium is very poor, we had to work out a procedure for determining the Se as SeH_2 by means of an hydride generator. The results we have thus far obtained are at this time not very satisfactory. The reason is that the selenium in the organic matrix is oxidized during our sample preparation procedure to the six-

valent state, which will form no hydride when treated with $NaBH_4$; therefore, the Se must have been already reduced to the tetravalent state. This procedure is not straightforward and the results will be published in another paper.

Acknowledgments

We wish to thank Dr. A. Aivasidis (Jülich) and Dr. G. Diekert (Marburg) for kindly providing cell material of *M. barkeri* grown on acetate and of *M. smithii* grown on H_2–CO_2, respectively. We are also grateful to Prof. Dr. H. Sahm for his aid. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

References

1. W. E. Balch, G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe, *Microbiol. Rev.* **43,** 260 (1979).
2. H. J. M. Bowen, *Trace Elements in Biochemistry,* Academic Press, New York, 1966.
3. G. Diekert, U. Konheiser, K. Piechulla, and R. K. Thauer, *J. Bacteriol.* **148,** 459 (1981).
4. A. A. Esener, J. A. Roels, and N. W. F. Kossen, *Biotechnol. Bioeng.* **24,** 1445 (1982).
4a. E. M. Godsy, *Appl. Environ. Microbiol.* **39,** 1074 (1980).
5. E.-G. Graf and R. K. Thauer, *FEBS Lett.* 136, 165 (1981).
6. D. Herbert, in *Microbial Reaction to Environment* (11th Symp. Soc. General Microbiol.), Cambridge University Press, Cambridge, UK, 1961, pp. 391–416.
7. H. Hippe, D. Caspari, K. Fiebig, and G. Gottschalk, *Proc. Natl. Acad. Sci. USA* **76,** 494 (1979).
8. T. J. Hutten, H. C. M. Bongaerts, C. van der Drift, and G. D. Vogels, *Antonie van Leeuwenhoek* **46,** 601 (1980).
9. K. F. Jarrell and G. D. Sprott, *Can. J. Microbiol.* **27,** 720 (1981).
10. K. F. Jarrell, J. R. Colvin, and G. D. Sprott, *J. Bacteriol.* **149,** 346 (1982).
11. J. B. Jones and T. C. Stadtman, *J. Bacteriol.* **130,** 1404 (1977).
12. G. E. Jones, L. G. Royle, and L. Murray, *Appl. Environ. Microbiol.* **38,** 800 (1979).
13. O. Kandler and H. König, *Arch. Microbiol.* **118,** 141 (1978).
14. T. W. Kirby, J. R. Lancaster, Jr., and I. Fridovich, *Arch. Biochem. Biophys.* **210,** 140 (1981).
15. F.-C., Kung, J. Raymond, and D. A. Glaser, *J. Bacteriol.* **126,** 1089 (1976).
16. J. K. Lanyi, *Biochem. Biophys. Acta* **559,** 377 (1979).
17. R. A. Mah, M. R. Smith, and L. Baresi, *Appl. Environ. Microbiol.* **35,** 1174 (1978).
18. R. A. Mah and M. R. Smith, in *The Prokaryotes* (M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel, eds.), Springer-Verlag, Berlin, 1981, pp. 948–977.
19. G. B. Patel, and L. A. Roth, *Can. J. Microbiol.* **23,** 893 (1977).
20. G. B. Patel, A. W. Khan, and L. A. Roth, *J. Appl. Microbiol.* **45,** 347 (1978).
21. H.-J. Perski, J. Moll and R. K. Thauer, *Arch. Microbiol.* **130,** 319 (1981).
21a. H.-J. Perski, P. Schönheit, and R. K. Thauer, *FEBS Lett.* **143,** 323 (1982).

22. N. Pfennig and K. D. Lippert, *Arch. Mikrobiol.* **55,** 245 (1966).
23. P. H. Rönnow and L. A. H. Gunnarsson, *FEMS Microbiol. Lett.* **14,** 311 (1982).
24. P. A. Scherer and R. K. Thauer, *Eur. J. Biochem.* **85,** 125 (1978).
25. P. Scherer and H. Sahm, in *Proceedings of the 1st International Symposium on Anaerobic Digestion,* Cardiff, 1979, Poster Papers (D. A. Stafford and B. I. Wheatley, eds.), A. D. Scientific Press, Cardiff, 1980, pp. 45–47.
26. P. Scherer and H. Sahm, *Eur. J. Appl. Microbiol. Biotechnol.* **12,** 28 (1981).
27. P. Scherer and H. Sahm, *Acta Biotechnologica* **1,** 57 (1981).
28. P. Scherer, M. Kluge, J. Klein, and H. Sahm, *Biotechnol. Bioeng.* **23,** 1057 (1981).
29. C. G. T. P. Schnellen, Thesis, Technical University of Delft, The Netherlands, (Rotterdam: De Maastad, publisher), 1947.
30. P. Schönheit, J. Moll, and R. K. Thauer, *Arch. Microbiol.* **123,** 105 (1979).
31. G. D. Sprott and K. F. Jarrell, *Can. J. Microbiol.* **27,** 444 (1981).
32. T. C. Stadtman, and B. A. Blaylock, *Fed. Proc.* **25,** 1657 (1966).
33. C. H. Suelter, in *CRC Handbook of Microbiology,* vol. 4, 2nd ed., A. I. Laskin and H. A. Lechevalier, eds., CRC Press, Boca Raton, Florida, 1982, pp. 553–564.
34. F. P. Takacs, T. I. Matula, and R. A. MacLeod, *J. Bacteriol.* **87,** 510 (1964).
35. G. D. Vogels, J. T. Keltjens, T. J. Hutten, and C. van der Drift, *Zbl. Bakt. Hyg. I. Abt. Orig. C* **3,** 258 (1982).
36. W. B. Whitman, E. Ankwanda, and R. S. Wolfe, *J. Bacteriol.* **149,** 852 (1982).
37. R. J. P. Williams, *Q. Rev.* **24,** 331 (1970).
38. C. R. Woese, *Sci. Amer.* **244**(6), 94 (1981).
39. E. A. Wolin, M. J. Wolin, and R. S. Wolfe, *J. Biol. Chem.* **238,** 2882 (1963).
40. S. Yamazaki and L. Tsai, *Fed. Proc.* **39,** p. 1698 (Abstr. 490) (1980).
41. S. Yamazaki and L. Tsai, *J. Biol. Chem.* **255,** 6462 (1980).