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Effect of carbon dioxide and hydrostatic pressure on the pH of culture media and the growth of methanogens at elevated temperature

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Summary. High pressure/high temperature investigations on thermophilic methanogens require specific precautions to provide well-defined pH conditions in their culture media. Applying CO_2 as carbon source, sufficient buffering capacity of the culture medium is of crucial importance in investigations involving elevated pressures. In order to separate pressure effects on the growth and reproduction of thermophilic methanogens from pressure-induced protonation/deprotonation and increased solubility of gaseous components, direct pH measurements in common culture media in the absence and in the presence of CO_2 were performed at elevated temperature (65° C), and at pressures up to 100 MPa. Neutral phosphate buffer at high pressure shows a significant downward shift of its pH which is strongly enhanced in the presence of CO_2. In minimal media containing acetate, carbonate, formate and phosphate in $\leqslant 100$ mM concentrations, 120 mM HEPES is found to provide optimum pH stability: near neutrality the pH change upon CO_2 saturation in the absence and in the presence of HEPES amounts to $\Delta pH = 2.10$ and 0.41, respectively; the corresponding pressure dependences are $\Delta pH/100$ MPa $= -0.26$ and -0.07. As taken from these results, the apparent pressure dependence of the optimum growth of *Methanococcus thermolithotrophicus* at 65° C in minimal medium reflects the pH shift below the cutoff point of growth (pH 5.5), rather than pressure-induced growth inhibition. At constant pH, elevated pressure up to 400 MPa is found to increase the rate and yield of growth; at the same time, alterations in the phenotype of the bacterium are observed.

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Introduction

The viability of microorganisms in their natural habitat depends in a complex way on the various environmental parameters. No general rules have been uncovered which would describe how temperature, pressure, pH and water activity cooperate in a synergistic or antagonistic way. What seems established is that the range of pH tolerance is narrowed with increasing hydrostatic pressure (Heefner 1982; Campbell et al. 1985). In defining the limits of temperature, Baross and Deming (1983) postulated the liquid state of water to be a necessary and sufficient requirement for life. Since elevated pressure favours the liquid state, one might expect that high pressure may broaden the temperature range of viability. In following this idea, hyperthermophilic microorganisms with optimum temperatures around 100° C (Stetter, 1985, 1986; Stetter et al. 1986) cause severe technical problems. These refer (i) to the corrosive effects of the gaseous sustrates (e.g. H_2 or H_2S), and (ii) to the problem of establishing well-defined growth conditions at high pressure and high temperature.

Concerning (i), high-pressure equipment for growing extreme thermophiles on gaseous substrates at high temperatures has been recently developed (Bernhardt et al. 1987). In connection with (ii), investigations on thermophilic methanogens require specific precautions, because CO_2 as common carbon source strongly promotes the down-shift in pH reported for phosphate, bicarbonate or neutral minimal media at elevated pressure (Neumann et al. 1973; Jaenicke 1983, 1987).

The solubility of CO_2 in water is affected by pressure and temperature in an antagonistic manner. Quantitative data in the range 0—50° C refer mainly to water and simple binary systems (Lan-

dolt-Börnstein 1962). The influence of electrolytes and organic substances in aqueous solution on the solubility of gases has been discussed by Schumpe et al. (1982). For complex microbial culture media with numerous components neither solubilities nor pH determinations are available. In common biochemical fermentation processes, the total pressure is usually not far from atmospheric pressure. Under this condition, Henry's law is sufficient to quantify gas solubilities. Under high pressure/high temperature conditions, no predictions can be made, especially because the increase in gas solubility is parallelled by the increased dissociation of weak acids, due to the electrostrictive effect of ion formation. For the given reasons, in the present paper an attempt is made to measure the effect of CO_2 and high hydrostatic pressure on the pH of a number of culture media at elevated temperature.

Materials and methods

Bacterial cultures

The thermophilic archaebacterium *Methanococcus thermolithotrophicus* has its temperature maximum at 70°C; under optimum temperature conditions, at 65°C, its doubling time is ca. 55 min. Inoculation of a 5% bacterial suspension in 20 ml medium was performed in serum flasks containing a gas mixture of 80% H_2 and 20% CO_2 at 0.2 MPa (Huber et al. 1982). For high pressure experiments, the inoculum was transferred into nickel tubes in an anaerobic chamber. Gas exchange and high pressure experiments were performed as described elsewhere (Bernhardt et al. 1987).

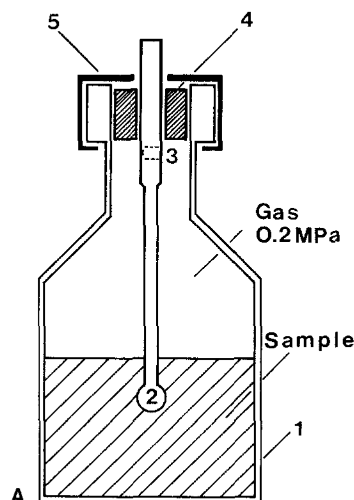
Media and substances

The following culture media were applied:
MGG: (minimal medium): 0.34 g KCl, 4.30 g $MgCl_2 \cdot 6H_2O$, 3.46 g $MgSO_4 \cdot 7H_2O$, 0.26 g NH_4Cl, 0.14 g $CaCl_2 \cdot 2H_2O$, 0.14 g K_2HPO_4, 18.0 g NaCl, 5.0 g $NaHCO_3$, 10 ml mineral salts, according to Balch et al. (1979), 1.0 ml $Fe(NH_4)_2(SO_4)_2$ (0.2%), 1.0 ml Resazurin (0.1%), 0.50 g $Na_2S \cdot 9H_2O$, with water ad 1000 ml.
MG⁻: MGG plus 0.5 g cysteine · HCl, 1.0 g sodium acetate and 10 ml vitamins, according to Balch et al. (1979).
MG: MG^- plus 1.0 g yeast extract, 1.0 g peptone.
MG^- HEPES: MG^- plus 120 mM HEPES.
MG^- PIPES: MG^- plus 54 mM PIPES.

Chemicals were obtained from Merck (Darmstadt). HEPES from Sigma (München), and cysteine · HCl from Fluka (Basel). Quartz bidistilled water was used throughout. The pH of the media was adjusted to pH 6.9 before autoclaving and gas exchange at room temperature.

Methods

For high pressure autoclaves, as well as equipment for pressurization and pressure/temperature control, c.f. Schade et al. (1980) and Bernhardt et al. (1987). pH profiles of bacterial growth were measured in serum flasks supplied with a microelectrode (Ingold, Frankfurt, type 405-M3) (Fig. 1A) connected to a digital pH-meter (Knick, Berlin, type 643). To monitor the EMF a W & W recorder (W & W Electronic Inc., Basel) was applied. The electrode was calibrated at 20 and 65°C using acetate and phosphate standard buffers at 0.2 MPa N_2 or H_2.



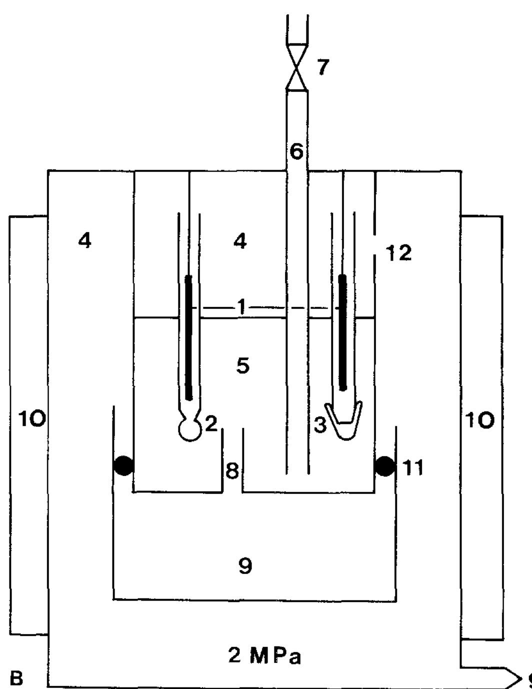
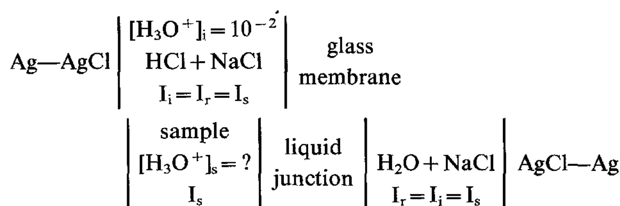


Fig. 1. A Device for pH determination under normal fermentation conditions: 65°C, 0.2 MPa H_2/CO_2. *1*, serum flask; *2*, pH electrode; *3*, bore-hole to equalize outside pressure; *4*, chlorobutyl cautchouk stopper; *5*, aluminum cap. **B.** Device for pH determination at high pressure in the presence of gases. *1*, Ag—AgCl; *2*, pH glass electrode; *3*, reference electrode; *4*, silicon oil; *5*, sample; *6*, gas supply; *7*, valve; *8*, connecting tube; *9*, pressure supply; *10*, heating system; *11*, O-ring; *12*, pressure balancing inlet

Altering the gases and the pressures (from atmospheric pressure to 0.2 MPa) had no effect on the pH; the same holds for Na_2S as an additive interacting with the electrode ($t \leq 6$ h).

pH measurements at high pressure and 65° C made use of an electrochemical cell designed for high pressure/high temperature work (Fig. 1B) (Distèche 1959, 1962, 1972, 1974; Dogan et al. 1987). The electrochemical description of the cell is as follows:



where I is the ionic strength, i, r, s refer to the pH glass electrode, the reference electrode and the sample to test, respectively.

Since all culture media contained 0.41 N Cl^-, the chloride concentration of both electrodes had to be adjusted to 0.41 N Cl^-: Reference electrode 0.41 N NaCl, glass electrode 0.41 N total Cl^- (containing 0.01 N HCl). This way the cell is built to minimize the junction potential and to give a highly stable electromotive force which permits to detect 0.001 pH variations. The calibration is made by measuring the asymmetry and the slope of the electrode at the ionic strength of the solution to test. This was accomplished by titrating a solution of Na_3PO_4—NaCl with HCl. The slope of the electrodes used was $S_1 = 57.93$ mV/pH unit at pH 2.0—7.2, and $S_2 = 57.25$ mV/pH unit at pH 7.2—8.5 (25° C). The corresponding temperature factors are $S_1/K = 0.194$ and $S_2/K = 0.192$, respectively. The absolute value of pH can be measured with an accuracy of 0.01 pH unit. The pH was calculated from the corrected electromotive force (EMF_c), i.e. the measured EMF minus the asymmetry potential, according to

$$pH = -\frac{EMF_c}{S} + 2 \qquad (1)$$

The temperature was measured using a two-terminal IC temperature transducer AD 590 (Analog Device).

In order to quantify bacterial growth at high temperature and high pressure, cell counting in a Neubauer chamber with 0.0025 mm^2 area and 0.02 mm depth (Zeiss phase-contrast microscope) was applied.

Results and discussion

The metabolic energy of *Methanococcus thermolithotrophicus* is based on the equation

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \qquad (2)$$

The stoichiometry of the reaction suggests the reaction volume to be negative, independent of the culture medium. In contrast to the expected enhancement of growth, elevated hydrostatic pressure is found to inhibit the growth of *Methanococcus* under optimum conditions, even at moderate pressure. As shown in Fig. 2, the limiting pressure where cell lysis outruns growth varies significantly in different culture media. The actual growth inhibition is even more dramatic, because differentiating intact cells and cell fragments is difficult so that the determination of cell numbers gives systematically too high values. It is reasonable to assume that at high pressure, where the cell number drops below the inoculation density, no intact cells are left. Applying culture media enriched by the addition of yeast extract, peptone, vitamins, sodium acetate and cysteine, the range of viability is shifted to higher pressures. Since part of the given additives act as weak buffers, one may assume that the increased stability is caused by restricting the pressure-induced acidification. In fact, *Methanococcus thermolithotrophicus* exhibits a significant growth inhibition at pH < 5.5 (cf. Fig. 3C).

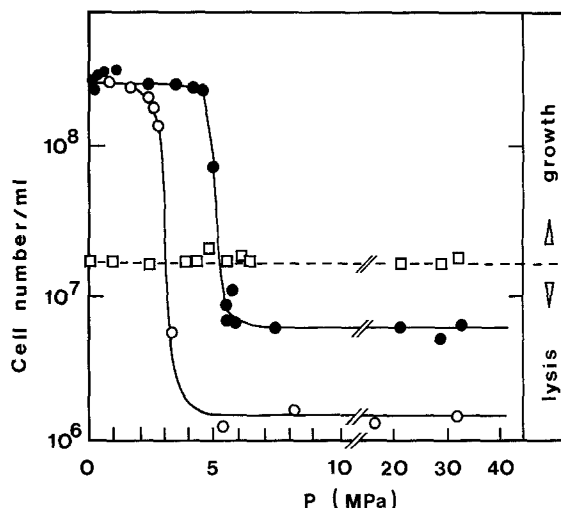


Fig. 2. Pressure effect on the growth of *Methanococcus thermolithotrophicus* in unbuffered standard media, MGG and MG. 4 ml inoculum (pH 6.9) plus 6 ml H_2/CO_2 (0.4 MPa) were incubated at 65° C during 20 h. pH was measured before the gas mixture was added. (○) MGG, (●) MG, (□) initial cell number before pressurization

In order to increase the buffer capacity, various buffer substances were investigated with respect to their influence on the growth of *Methanococcus thermolithotrophicus* at elevated temperature. Imidazole turns out to inhibit the growth even at low concentration (25 mM, pH 6.9). Since most organic buffer components show high enthalpies of ionization, i.e. a strong temperature dependence of their pH, PIPES and HEPES were chosen as promising candidates. Their characteristics are

PIPES pK_a (20° C) = 6.80 $\Delta pK_a/K = -0.0085$
HEPES pK_a (20° C) = 7.55 $\Delta pK_a/K = -0.014$

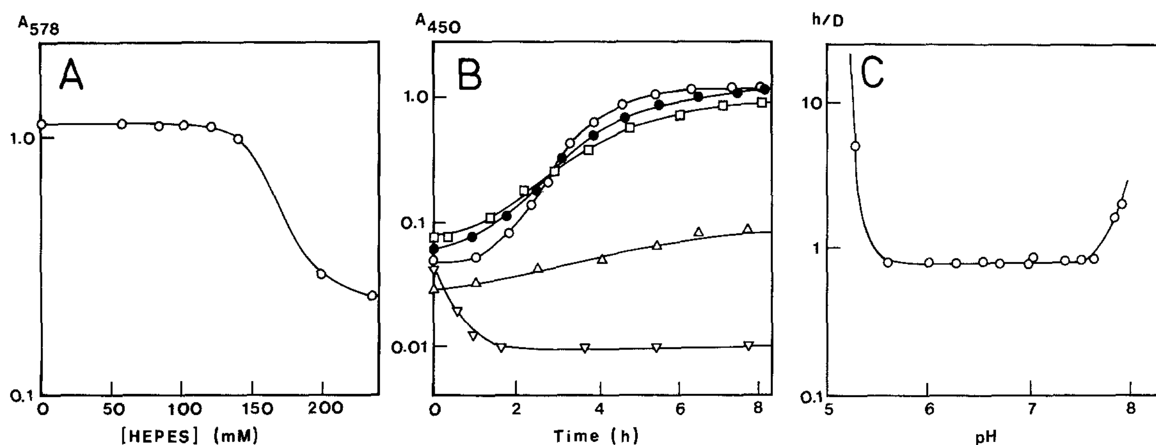


Fig. 3. Influence of solvent conditions on the growth of *Methanococcus thermolithotrophicus*. **A.** Effect of HEPES on the growth of *Methanococcus thermolithotrophicus* at 65° C, 0.2 MPa H_2/CO_2. 20 ml inoculum (0.045 OD in MG^-) were incubated in serum flasks at given HEPES concentations during 8 h. $A_{578\,nm}$ was measured to monitor the limiting cell population under stationary conditions. **B.** Cell proliferation of *Methanococcus thermolithotrophicus* at varying pH (65° C, 0.2 MPa H_2/CO_2). In order to vary the pH of the culture medium MG^- was titrated using 50% H_2SO_4 and half-saturated NaOH, respectively. (▽) pH 4.5; (△) pH 5.2; (○) pH 5.6–6.6; (●) pH 7.0; (□) pH 7.9. **C.** pH effect on the doubling time of *Methanococcus thermolithotrophicus*. Ordinate in hours per doubling

(Westcott 1978). Due to its low solubility, PIPES does not provide sufficient buffer capacity.

As shown in Fig. 3A, HEPES under standard conditions (0.2 MPa H_2/CO_2, 65° C) does not affect the proliferation of *Methanococcus thermolithotrophicus* at concentrations below 125 mM. Figure 3B depicts the time dependent growth of the bacterium at varying pH, while Fig. 3C illustrates the pH dependence. The doubling times were measured after titrating the culture medium with 50% H_2SO_4 or half-saturated NaOH. Reference experiments without inoculum showed a maximum pH-shift of 0.3 pH units, after 8 h incubation at 0.2 MPa H_2/CO_2, 65° C.

Table 1. Solubility of CO_2 in water and aqueous salines at 0 and 60° C[a]

P_{extern} (MPa)	P_{CO_2} (MPa)	L (ml CO_2/ml solution)		
		water		buffer solution[b]
		60° C	0° C	0° C
0.1	0.02	0.27	0.14	0.11
1.0	0.2	0.67	4.5	3.0
2.0	0.4	1.34	8.5	5.2
2.5	0.5	1.68	10.0	6.5

[a] Data taken from Landolt-Börnstein (1962), Stephen & Stephen (1963)

[b] To simulate the present experimental conditions, values for artificial seawater concentrate with 103 g salt/kg have been chosen

Increased external pressure leads to increased solubility of H_2/CO_2. At 30° C, the 80% H_2/20% CO_2 mixture follows Henry's law up to ≈3 MPa (Landolt-Börnstein 1962). For the given experiments, the maximum CO_2 concentration was $L_{CO_2} = 1.87$ cm^3 CO_2/cm^3 H_2O. At 65° C, this concentration is reached at an external pressure of $p \approx 3$ MPa, as interpolated from data summarized in Table 1, and from direct pH measurements illustrated in Fig. 4B.

Saturation of aqueous culture media with CO_2 may cause drastic pH changes. As indicated in Table 2, the pH shift in unbuffered minimal medium at 65° C exceeds 2 pH units. With increasing external pressure, in the presence of CO_2, a further decrease in pH is observed (Fig. 4B); ΔpH/100 MPa amounts to ≈ -0.3 pH units. In the presence of HEPES or PIPES, this effect is strongly diminished (Table 2), so that high pressure/high temperature experiments in the presence of CO_2 are rendered possible without excessive alterations in pH.

Correlating the previously mentioned pH changes with the pH profile of the growth of *Methanococcus thermolithotrophicus* (Fig. 3C), it is obvious that the apparent medium effects illustrated in Fig. 1 refer to the combined effects of pressure and pH: the differences in the growth vs pressure curves in different media are predominantly caused by the pH-shifts at saturating CO_2 concentrations. The presence of H_2 does not affect the pH of the media to a significant extent.

Based on (i) the known pH shifts in culture

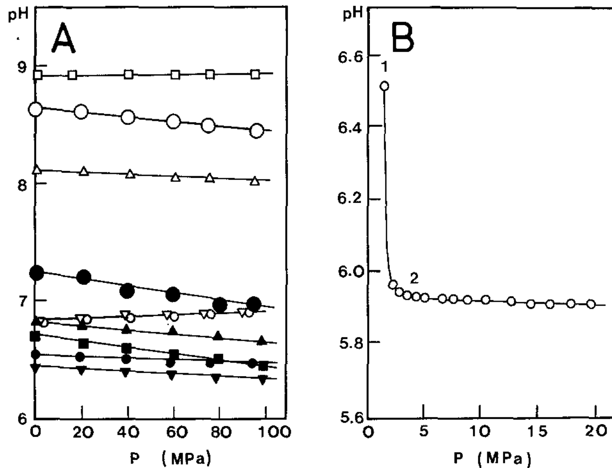


Fig. 4. Pressure effect on standard media at 65° C in the absence and presence of CO_2. **A.** Pressure effect on the pH of MGG (○, ●), Mg (△, ▲), MG^- (□, ■), MG^- PIPES (▽, ▼) and MG^-HEPES (o, •). *Open symbols*, in the absence of CO_2; *closed symbols*, in the presence of ≈ 14 μMol · ml^{-1} CO_2. **B.** Effect of CO_2 on the pH of MG^-HEPES buffer at 65° C. 40 ml buffer were preincubated at 2 MPa; after reaching a stable EMF, addition of 20 ml CO_2 at 3 MPa (1) causes a pH shift of ≈0.6 pH units. A further increase of hydrostatic pressure results in a marginal pH shift only (2)

Table 2. Effect of CO_2 and hydrostatic pressure on the pH of growth media at 65° C

Medium[a]	pH		ΔpH[c]	ΔpH/100 MPa	
	$-CO_2$	$+CO_2^b$		$-CO_2$	$+CO_2^b$
MGG	8.65	7.25	1.40	−0.13	−0.30
MG	8.11	6.86	1.25	−0.09	−0.23
MG^-	8.80	6.70	2.10	+0.04	−0.26
MG^-HEPES	6.82	6.41	0.41	+0.06	−0.07
MG^-PIPES	6.84	6.52	0.32	+0.05	−0.07

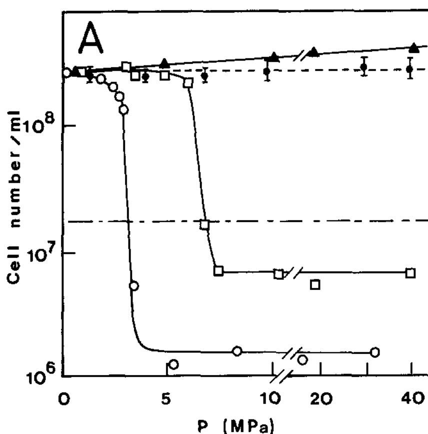
[a] cf. *Materials and methods*
[b] max CO_2 concentration 14 μMol · ml^{-1}
[c] Decrease of pH after adding 14 μMol · ml^{-1} CO_2

media at high pressure and high temperature in the presence of H_2/CO_2, and (ii) the pH profile of growth, given in Fig. 3C, the pressure dependence of the cell number of *Methanococcus thermolithotrophicus* at optimum pH can now be established. As shown in Fig. 5A, high pressure in the range of up to 40 MPa enhances cell proliferation, provided the pH does not drop below pH≈5.6. In unbuffered (MGG) and insufficiently buffered (PIPES) media, the downward shift of the pH leads to the above mentioned apparent pressure inhibition at 3 and 7 MPa, respectively. At $p>40$ MPa, and constant pH (HEPES), elongation and other morphological alterations of the cells become visible, and beyond 100 MPa, lysis occurs (Fig. 5B).

Conclusions

The metabolic energy of *Methanococcus* is essentially based on two gaseous substrates both of which cause specific problems in high pressure/high temperature experimentation. Handling hydrogen at $p \leq 200$ MPa and >60° C causes severe permeability problems which have been successfully addressed by applying nickel tubes as containers for bacterial growth (Bernhardt et al. 1987). Carbon dioxide, together with high pressure ionization of common standard buffers, leads to a significant downward shift of the pH of growth media which may cause growth inhibition.

Since no pH values for typical growth media



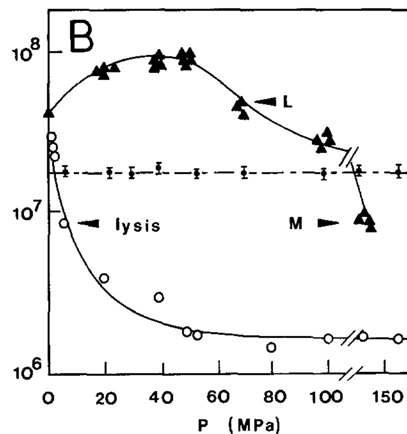


Fig. 5. Pressure effect on the growth of *Methanococcus thermolithotrophicus* in unbuffered and buffered minimal media at 65° C. **A.** Bacterial growth at moderate pressures. (○) MGG; (□) MG^- plus 54 mM PIPES; (▲) MG^- plus 120 mM HEPES; (Ī) initial cell number: controls without external pressure. Conditions as in Fig. 1. **B.** Bacterial growth at high hydrostatic pressure. (○) MG^-; (▲) MG^- plus 120 mM HEPES. Conditions as in Fig. 1, except 5h incubation (Ī) initial cell number. L, anomalously elongated large cells; M, "mini cells" (vesicles?)

at high pressure and high temperature have been tabulated, the respective data had to be determined before high pressure effects on the growth of methanogenic bacteria at high temperature could be approached. For *Methanococcus thermolithotrophicus*, optimum buffer conditions were obtained using MG^- medium (Balch et al. 1979), supplemented with 120 mM HEPES. Measurements of the growth rate of the bacterium in this medium exhibit biphasic characteristics: Below 40 MPa, cell proliferation is enhanced without detectable alterations in the size and shape of the cells. Beyond 40 MPa, elongated, large sized cells are formed. Similar anomalies have been reported for a number of microorganisms exposed to high pressure at ambient temperature (ZoBell and Cobet 1964). Further increase of pressure finally causes cell lysis, again confirming previous observations (ZoBell 1970). The question whether high pressure may extend the range of viability of thermophilic organisms cannot be answered by the present experiments. Studies devoted to this question are in progress.

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