Methane as a Minor Product of Pyruvate Metabolism by Sulphate-reducing and Other Bacteria

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SUMMARY

Disrupted cells of some *Desulfovibrio* species, of *Desulfotomaculum ruminis* and of certain other anaerobes produced methane as a minor product of pyruvic phosphoroclastism. In one *Desulfovibrio* species the reaction, which was not specially sensitive to air, involved vitamin B₁₂, co-enzyme A, thiamine pyrophosphate, magnesium ions and acetyl phosphate. Adenine and other nucleotides stimulated the reaction; a mixture of ATP and AMP was most effective. Methionine stimulated the reaction but the other methyl donors did not. In optimal conditions methane accounted for 0.1 to 0.02 mole % of the pyruvate metabolized, formed at 20 to 30 nl. CH₄/mg. bacterial protein/hr. Tests in D₂O indicated that the methane came from the methyl-carbon of pyruvate; correspondingly, ethane formation from α -ketobutyrate was detected.

INTRODUCTION

Sisler & ZoBell (1951) reported that certain cultures of marine sulphate-reducing bacteria, pure by normal criteria, formed methane during growth in lactate + sulphate media. Though occasional references to this finding have appeared, and Sorokin (1956) failed to confirm it, interest has tended to centre on the disputed question of the oxidation of methane (and other hydrocarbons) by desulfovibrios (e.g. Sorokin, 1957; Davis & Yarbrough, 1966). Methane formation by phosphoroclastic preparations from strains of sulphate-reducing bacteria was reported briefly by Postgate (1969). The present paper describes the reaction in one *Desulfovibrio* strain in more detail and reports its occurrence in other bacteria.

METHODS

Cultivation. *Desulfovibrio desulfuricans* variety *azotovorans*, strain BERRE S (NCIB 8388) was grown at 30° in a 600 ml. continuous-culture apparatus (Baker, 1968) at dilution rates ranging from 0.05 to 0.1 hr⁻¹. The medium used was deficient in fixed nitrogen; it contained (g./l.): KH₂PO₄, 0.5; Na₂SO₄, 4.5; CaCl₂·2H₂O, 0.06; MgSO₄·7H₂O, 0.06; FeSO₄·7H₂O, 0.004; sodium lactate, 6; sodium citrate, 0.3 (to prevent precipitation of metal hydroxides) in distilled water; plus 10 ml. of a trace element mixture containing (mg.): H₃BO₃, 2.32; CoSO₄·7H₂O, 95.6; CuSO₄·5H₂O, 8; MnSO₄·4H₂O, 8; NaMoO₄·2H₂O, 30; ZnSO₄·7H₂O, 174 in 1 l. distilled water. The complete medium was at pH 8.2; the atmosphere was N₂ containing 6 to 8% (v/v) CO₂. The CO₂ buffered the culture to between pH 7.6 and 8, and displaced excess sulphide.

Effluent was harvested when 0.75 to 3 l. had accumulated, the organisms washed by centrifugation in 25 mM-tris buffer (pH 7.5 ± 0.1) and disrupted by dropping a cream of washed bacteria (equiv. 50 to 100 mg. dry wt./ml. tris buffer) into liquid nitrogen. Batch cultures of BERRE S and other strains of sulphate-reducing bacteria were grown in long-necked 250 or 500 ml. flasks of medium C (Postgate, 1966) under N_2 and were disrupted in a similar manner.

Criteria of purity. The strains of sulphate-reducing bacteria were examined microscopically for morphological homogeneity and were tested for both aerobic and anaerobic contamination by using the conditions prescribed by Postgate (1953). No contaminants were found. These procedures might not reveal as contaminants methane-producing bacteria, which are known to associate with sulphate-reducing bacteria (e.g. Stadtman & Barker, 1951) and which could, if present, have been responsible for the phenomenon being investigated. The continuous culture of strain BERRE S, the organisms routinely studied, was therefore examined exhaustively for methane bacteria, by using the media of Stadtman & Barker (1951) with sodium acetate (the main product after growth in the continuous culture), sodium formate, sodium pyruvate or ethanol (0.1%) as carbon sources. Neither 'shake' cultures in such media solidified with agar, nor anaerobic enrichment cultures in stoppered bottles, showed bacterial growth or gas production at 30°. The culture was therefore accepted as free from methane bacteria.

Enzyme assays. Pyruvic phosphoroclastic activity was detected by conventional Warburg manometry at 30°. The preparation under test was set up in a manometer vessel without KOH, gas evolution in response to added pyruvate was observed and finally CO_2 was demonstrated in the gas mixture by introducing 10 N-KOH and observing gas absorption. Methane formation was detected by vapour-phase chromatography, by the following routine procedure. 'Penicillin vials' of about 8 ml. capacity, cooled in ice, were provided with 0.5 to 1 ml. tris buffer containing all necessary co-factors (see Results) except for $MgCl_2$ and the substrate pyruvate. A fresh frozen-cell preparation, after 5 to 20 min. in liquid N_2 , was thawed with 1 μ g. each of RNase and DNase under nitrogen or argon to between 0 and 10° and homogenized by vortex-stirring with a Whirlimixer (Scientific Industries (U.K.) Ltd.). Volumes of cell preparation equal to the volumes of co-factor solution already present were distributed among the penicillin vials, usually to give equiv. 8 to 15 mg. bacterial protein/vessel. While still cool the vials were flushed with argon and the reaction initiated as soon as possible by injecting pyruvate + $MgCl_2$, transferring the vials to a thermostat bath at 30° and shaking gently (80 to 85 strokes/min. at amplitude 4 cm.). In a few experiments, nitrogen or other gases replaced argon; in some the reaction system was set up complete with substrates and initiated by warming to 30°; in testing new organisms a control without pyruvate was included. Protein was estimated by the biuret method with serum albumin as standard.

Gas chromatography. The gas phase was sampled by filling a syringe with 0.5 ml. argon or other appropriate gas, injecting this and, after a moment for mixing, removing 0.5 ml. for chromatography. CH_4 was detected and estimated on the Pye 104 gas-liquid chromatograph, with a 5 ft. (152 cm.) 'Porapak R' column at 75° or 45°, leading to a flame-ionization detector. The attenuation settings used were 10 to 50. The gas was identified as CH_4 by infrared spectroscopy in a specially scaled-up experiment. In time-course experiments a correction was made for losses and dilution

of CH_4 involved in the sampling procedure; the correction was imprecise because CO_2 and H_2 , which were produced simultaneously, altered the pressure within the reaction vessel by amounts which varied according to the conditions. In such experiments, therefore, only wide differences in rates of gas production were taken as meaningful.

The argon used contained small amounts of methane (0.2 to 1 part/ 10^8 , v/v) which were corrected for; the nitrogen contained none. The argon also contained a similar amount of an unidentified gas running just after hydrogen; though the chromatography detector was a hydrogen flame, very large amounts of hydrogen gave a signal just before the argon impurity peak, and the appearance of such a 'spike' during experiments was a useful indication that a conventional pyruvic phosphoroclastism was in progress.

For the separation of CD_4 from CH_4 a 5 Å molecular-sieve column (5 ft., 152 cm., 60 to 80 mesh, from F. & M. Scientific Ltd.) at 25° replaced the 'Porapak R' column. CD_4 (prepared from methyl isocyanide and *Azotobacter nitrogenase*; Kelly, Postgate & Richards, 1967) ran ahead of CH_4 and was distinguished from it unequivocally by cutting out and superimposing the traces.

Materials. Ordinary chemical reagents were of analytical grade, except some of those used in preparing media. Adenine- and other nucleotides, co-enzyme A, thiamine pyrophosphate, lithium acetyl phosphate, cyanocobalamin, thiocetic acid, sodium α -ketobutyrate and sodium pyruvate were Sigma products; the ADP contained variable amounts of AMP and was specially purified chromatographically for critical tests.

RESULTS

Conditions for methane formation. Suspensions of whole bacteria of *Desulfovibrio desulfuricans* strain BERRE S, harvested from continuous culture with gaseous N_2 as its sole nitrogen source, showed vigorous phosphoroclastic activity. Traces of methane were detected in the overlying gas amounting to 1 or 2 parts/ 10^8 formed in 3 hr. Methane was not detected above cultures of this strain when growing in sodium lactate media, nor did the strain utilize methane. No growth in excess of the control occurred when medium C without lactate was inoculated and incubated for 18 days at 30° under 1.5 volumes of $\text{N}_2 + 7\%$ (v/v) CH_4 . 'Blank' growth on the yeast extract in medium C indicated that these conditions were not intrinsically inimical to growth; no utilization of the CH_4 in the atmosphere was detected chromatographically.

Bacterial suspensions disrupted as described in Methods showed much more impressive methane production which varied according to test conditions; suspensions washed with distilled water and dried at 30° *in vacuo* were also active. Methane was also produced in Na phosphate, tris and Na dimethylglutarate buffers (25 mM, pH 7.5). Bacteria grown in medium C, which contained fixed nitrogen, then frozen and thawed as described in Methods, also gave active preparations. Bacteria from the continuous culture were used in most experiments because they were readily available.

Effects of co-factors. Yates (1967) showed that the phosphoroclastic system of strain BERRE S, like those of certain clostridia (Wiesendanger & Nisman, 1954; Biggins & Dilworth, 1968), was stimulated by ATP. Figure 1 illustrates stimulation of methane formation by ATP. Acetyl phosphate occasionally stimulated the system; Yates routinely added certain known co-factors of the phosphoroclastic system;

the following co-factors stimulated methane production by BERRE S preparations in 25 mM tris pH 7.5 (optimal concentrations are given in parentheses): MgCl_2 (2.5 mM), KH_2PO_4 (1.6 mM), co-enzyme A (0.5 mg./ml.), thiamine pyrophosphate (0.05 mg./ml.), acetyl phosphate (0.5 mg./ml.), ATP (1 mM). The concentration of co-enzyme A required for maximum activity was unexpectedly large but was indeed necessary; it could not be replaced by equimolar cysteine. No unequivocal effect of α -lipoic acid (0.5 mg./ml.) was detected; K^+ as KCl (5 mM) did not affect the reaction. Ferredoxin, rubredoxin and cytochrome c_3 extracted from strain BERRE S were tested as concentrates after ion-exchange chromatography (50 to 55 μg . carrier/ml.): ferredoxin and rubredoxin depressed methane production slightly; cytochrome c_3 had no effect. No clear effect on hydrogen production by any of these factors was observed.

Fig. 1. Effect of ATP on methane production by *Desulfovibrio desulfuricans* strain BERRE S disrupted by freezing in liquid nitrogen. Reaction mixture (2 ml.) contained acetyl phosphate, co-enzyme A, cyanocobalamin, thiamine pyrophosphate and potassium phosphate in tris buffer and equiv. 8.1 mg. bacterial protein at 30° under argon in an 8 ml. penicillin vial; for details see text. ○, ATP (1 mM), MgCl_2 (2.5 mM) and sodium pyruvate (7.5 mg./ml.) added to start reaction; ●, ATP omitted; ◐, pyruvate omitted. A boiled control showed no activity.

Blaylock & Stadtman (1963, 1964) showed that a methane-producing system from *Methanosarcina barkerii* utilized cobalamin derivatives and that methyl B_{12} was an intermediate. Cyanocobalamin stimulated methane production by the BERRE S preparation and showed optimal activity at 5 to 50 μg ./ml.; 500 μg ./ml. depressed activity by about 60%. During the reaction the pink B_{12} colour became bleached, suggesting that reduced intermediates capable of accepting methyl groups were being formed.

On the basis of these findings a standard reaction mixture was formulated for other

tests containing ATP, 1 mM; KH_2PO_4 , 2.5 mM; thiamine pyrophosphate, 0.1 mg./ml., co-enzyme A, 1 mg./ml.; lithium acetyl phosphate, 1 mg./ml.; cyanocobalamin, 0.01 mg./ml. tris buffer (0.025 M, pH 7.5). Sodium pyruvate (15 mg./ml.) + MgCl_2 (5 μmole) were generally injected in 0.1 ml. buffer to initiate reaction; in certain tests ATP or substitute nucleotides were omitted from the reaction mixture and injected with the substrates.

Effect of pH value, pyruvate and enzyme concentration. Methane was produced more rapidly at pH 7.5 than at pH 7 or 8. Pyruvate was necessary for methane formation (Fig. 1). Between 10 and 100 mg. sodium pyruvate/ml. the rate of production of CH_4 decreased, though the final amount did not necessarily do so; the initial rate was constant between 1 and 10 mg. pyruvate/ml. but the rate always decreased with time (see Fig. 1). The reaction rate with 5 mg. pyruvate/ml. was proportional to 'enzyme' concentration between equiv. 7 and 25 mg. bacterial protein/ml. but decreased at 35 mg./ml.

Effect of air. Extracts of *Methanobacterium omelianskii* are extremely sensitive to air (Wolin, Wolin & Wolfe, 1963). Preparations of *Desulfovibrio desulfuricans* strain BERRE S were not unduly sensitive: equiv. 6.3 mg. bacterial protein/vessel (2 ml. fluid) yielded 12.6 to 17.4 nl. CH_4 /mg. protein/hr under air, nitrogen + air to 0.02 atm. O_2 , or pure N_2 . CO_2 was formed in all conditions. A preparation of equiv. 8.5 mg. protein/ml. retained 65 % of the activity of a control in argon after stirring vigorously in air for 1 min. with a vortex mixer. This relative insensitivity to oxygen makes it unlikely that the stimulating effect of cyanocobalamin was due to scavenging of residual oxygen by the catalytic effect of vitamin B_{12} on thiol oxidation (Peel, 1963). Nevertheless, this possibility was tested by using sodium dithionite to remove oxygen; cyanocobalamin stimulated the reaction equally well whether $\text{Na}_2\text{S}_2\text{O}_4$ (0.5 mg./ml.) was present or not.

Effects of reaction products. The major products of pyruvic phosphoroclastism in desulfovibrios are acetate (by way of acetyl phosphate), CO_2 , H_2 and/or ethanol (Millet, 1954; Sadana, 1954). Acetyl phosphate yielded no methane alone but, in the presence of pyruvate, was a strong stimulant of CH_4 production. It was routinely present in the reaction mixtures. Sodium acetate (0.5 mg./ml.) had no effect; phosphoenol pyruvate (0.5 mg./ml.) was also inactive. CH_4 was formed under helium or N_2 as well as under argon, but an atmosphere of H_2 decreased the rate of production and yield by about 55 %. CO_2 , either 3 % (v/v) in the atmosphere or as 0.5 mg. NaHCO_3 /ml. in the reaction mixture, decreased the amounts of methane formed by 60 to 75 %. Ethanol (0.5 mg./ml.) inhibited methane production by 95 %.

Other substrates and inhibitors. Sodium formate, which undergoes the hydrogenlyase reaction in *Desulfovibrio* (Ishimoto, Yagi & Shiraki, 1957), yielded no methane at 0.5 mg./ml. Sodium α -ketobutyrate was metabolized by preparations of *D. desulfuricans* strain BERRE S with a nominal Q_{CO_2} of 4.8 $\mu\text{l. CO}_2$ /mg. bacterial protein/hr as compared with a control Q_{CO_2} value of 2.14 without substrate and of 40 with pyruvate (for calculating these values all the gaseous product was taken to be CO_2 though, with pyruvate, some was H_2); α -ketobutyrate depressed the 'blank' methane production by such preparations but ethane was detected in the overlying gas, formed at about 0.6 nl./mg. bacterial protein/hr. Methanol or propanol (0.5 mg./ml.) decreased the rate and amount of methane formation by 58 % and 90 %, respectively; butanol, at a similar concentration, had no influence on the reaction. Methylviologen (0.25 mM) completely

inhibited methane formation but stimulated formation of hydrogen. Methylviologen has a comparable effect on methane formation by washed *Methanobacterium omelian-skii* (Wolin, Wolfe & Wolin, 1964).

Effects of biological methylating agents. Choline chloride (0.5 mg./ml.) had no effect on methane formation. Methionine (0.05 to 1 mM) stimulated the reaction moderately in five tests out of six; S-adenosyl methionine (0.5 mg./ml.), which might have been formed from ATP and methionine, had no effect when tested as the iodide, in place of both ATP and methionine.

Effects of nucleotides. Reaction mixture made up without ATP was tested with ADP or AMP at 1 mM or pairs of adenine nucleotides at 0.5 mM each. For these tests specially purified ADP was used. In four separate tests, no single nucleotide was more effective than ATP and ADP was generally the least effective. But mixtures of nucleotides had profound effects, though test-to-test variations were considerable. ATP + AMP had maximum effect on one occasion but did not exceed ATP alone on another. Equimolar amounts of other nucleotide triphosphates replaced ATP: guanosine triphosphate and uridine triphosphate were about as effective as ATP, cytidine triphosphate showed 50 % activity, inosine triphosphate was inactive.

Table 1. *Stoichiometry of methane production by Desulfovibrio desulfuricans strain BERRE S during pyruvic phosphoroclasms*

Washed organisms were disrupted in liquid N₂ and incubated under argon in Warburg manometers at 30° with 7.5 to 10 mg. sodium pyruvate/ml. reaction mixture (see text). Seven different bacterial preparations are recorded.

mg. bacterial protein/ml.	Incubation (min.)	CO ₂ (μmole)	H ₂ (μmole)	CH ₄ (nmole)	CH ₄ as mole % CO ₂
35*	23	8.8	9.3	4.7	0.053
24	30	16.1	—	38.0	0.24
50	45	12.4	15.5	7.0	0.56
33†	50	5.0	0.56	7.2	1.4
9.2} ‡	55	9.1	2.2	0.48	0.053
9.2} ‡	65	7.05	4.5	0.38	0.051
7	110	11.4	0.7	2.87	0.025
7.7§	155	20.4	0.75	2.57	0.0126

* 0.5 mg. DL-methionine/ml. reaction vessel present.

† Bacteria grown with fixed-nitrogen (note relatively low CO₂ and H₂ output).

‡ Same bacterial preparations.

§ Experiment in penicillin vial, not Warburg manometer; all gases analysed by gas chromatography.

Stoichiometry. Blaylock & Stadtman (1963) noted that a considerable consumption of pyruvate was required to generate relatively little methane by extracts of *Methanobacterium barkeri*. Preparations of *Desulfovibrio desulfuricans* strain BERRE S were studied in 2-side arm Warburg flasks with a 'Suba-seal' closure on one arm to permit removal of gas samples and injections of alkali for gasometric CO₂ analysis. Table 1 quotes selected results from eight experiments and illustrates three points. (1) Methane was formed in greater proportions early in the reaction. (2) The theoretical CO₂:H₂ ratio of 1 for pyruvic phosphoroclasms was rarely obtained, and the H₂ yield was generally low. This finding was checked by experiments using gas-chromatographic analysis for CO₂ and H₂ with a gas-density detector: the situation was attributed to

hydrogen consumption by endogenous hydrogen acceptors in the strongly proteinaceous preparations necessary for this work: freshly disrupted strain BERRE S preparations had initial $-Q_H$ values of about 16 μ l. H_2 /mg. protein/hr. (3) Methane accounted for between 0.02 and 0.2 mole % of the pyruvate utilized, considerably less than the 3 to 7 mole % methane which Blaylock & Stadtman (1963) observed with *Methanosarcina barkerii* and methyl B_{12} as co-factor.

Origin of the methane. The experiments in which ethane was formed from α -keto-butyrate suggested that the ethane originated from the alkyl chain of the substrate, not by CO_2 reduction. This conclusion was checked by tests in D_2O , which would give CD_4 by a CO_2 -reduction mechanism but CH_3D (chromatographically indistinguishable from CH_4 in my test system) by a reductive decarboxylation mechanism. Bacteria pre-equilibrated 20 min. at 30° with D_2O buffer and vacuum-dried bacteria re-constituted in D_2O buffer were tested; both yielded peaks indistinguishable from CH_4 . Insufficient gas was found for identification of CH_3D by infrared spectroscopy.

Table 2. *Methane-producing activity of disrupted sulphate-reducing and other bacteria*

Washed organisms were disrupted in liquid N_2 and shaken under argon at 30° with 5 to 20 mg. sodium pyruvate/ml. reaction mixture (see text). Bacterial protein 3 to 30 mg./vessel according to activity; negative or 'trace' tests ran for 90 to 180 min., positives ran for 30 to 50 min. All preparations showed CO_2 and/or H_2 production from pyruvate; *Clostridium pasteurianum* and *Bacillus polymyxa* were not tested.

Species and strain with N.C.I.B. number	CH_4 produced (nl./mg. protein/hr.)
Sulphate-reducing bacteria	
<i>Desulfovibrio desulfuricans</i> BERRE S (8388)	15-20
<i>D. africanus</i> BENGHAZI (8401)	0.16
<i>D. salexigens</i> BRITISH GUIANA (8403)	1.7
<i>Desulfotomaculum ruminis</i> COLEMAN 42 (10,149)	9.5-11
Others	
<i>Clostridium pasteurianum</i> strain w	2.65

Methane at less than 0.1 nl./mg. hr. was formed by *Desulfovibrio desulfuricans* BERRE E (8387), *Desulfovibrio vulgaris* 'HILDENBOROUGH' (8303), *Desulfovibrio gigas* (9332), *Klebsiella pneumoniae* and *Bacillus polymyxa*. No methane was formed by *Desulfovibrio desulfuricans* 'ESSEX 6' (8307) or NORWAY 4 (8310), nor by *Rhodospirillum rubrum*.

Tests of other bacteria. Batches (250 ml.) of sulphate-reducing bacteria were grown in medium C under nitrogen, harvested and disrupted in liquid nitrogen as described. The preparations were tested in the usual reaction mixture for methane formation, and pyruvic phosphoroclastic activity was tested by Warburg manometry. Table 2 lists the strains tested; activity was not restricted to any particular species or even genus and not all strains of a species were active. Some were tested twice and showed wide differences; only *Desulfotomaculum ruminis* approached the activity of the BERRE S strain of *Desulfovibrio desulfuricans*. Non-sulphate-reducing bacteria were obtained from Dr M. Kelly (this laboratory) as live cultures or frozen suspensions grown in conventional nitrogen-deficient media for 2 to 4 days at 30° ; phosphoroclastic activity was obvious in the preparation of *Clostridium pasteurianum* because a 'hydrogen spike' (see Methods) was observed in the chromatographic tests. The presence of

chalk carried over from the culture medium precluded a satisfactory manometric test for pyruvic phosphoroclastism with this strain and *Bacillus polymyxa*. *Clostridium pasteurianum* was tested with sodium α -ketobutyrate, from which it formed a trace of ethane.

DISCUSSION

Methane is unequivocally a minor product of pyruvic phosphoroclastism in a majority of the strains of sulphate-reducing bacteria tested and cannot reasonably be attributed to contaminant methane bacteria. It is produced in insufficient amounts to account for the stimulation of pyruvic phosphoroclastism in *Desulfovibrio desulfuricans* strain BERRE S as recorded by Yates (1967). The strains differed in their productivities, as Sisler & ZoBell (1951) reported among the cultures they surveyed, and though amounts of methane comparable with Sisler & ZoBell's maximum yields were not encountered over live cultures, it is reasonable to suppose that differences in strains and test conditions used account for this discrepancy and for Sorokin's (1956) failure to confirm Sisler & ZoBell's report.

A soluble methane-producing system was not studied because attempts to extract a soluble pyruvic phosphoroclastic system from *Desulfovibrio desulfuricans* strain BERRE S have not so far been successful. The properties of the particulate preparations, though not always as clear-cut as might be expected from soluble enzymes, resemble in certain details those of the extracts of *Methanosarcina barkerii* studied by Blaylock & Stadtman (1963, 1964; see also Stadtman, 1967): vitamin B₁₂ is involved (it stimulates and becomes bleached during the reaction), and adenine nucleotides stimulate. But the source of the methane is the 3-C group of pyruvate, which it is not in extracts of *M. barkerii*. As in true methane bacteria, the yield of methane is low in terms of pyruvate consumed. This feature also recalls the low ammonia yield in the cell-free nitrogen-fixing phosphoroclastic system from *Clostridium pasteurianum* studied by Carnahan, Mortenson, Mower & Castle (1960), but there seems to be no direct connexion between methane formation and nitrogen fixation, since (1) ammonia-grown populations of *D. desulfuricans* strain BERRE S formed methane, (2) non-nitrogen-fixing strains such as *Desulfovibrio salexigens* formed methane, (3) several nitrogen-fixing anaerobes did not form methane. The yield of CH₄ from pyruvate with strain BERRE S was 15 to 150 times lower than that recorded for *M. barkerii*; it might have been improved had methyl B₁₂, the functional co-factor in *M. barkerii*, been available, but this reaction is clearly a small-scale process in physiological terms. A major difference between the *Desulfovibrio* system and that in a true methane bacterium is its relative insensitivity to oxygen. Minor differences include activity of acetyl phosphate, not reported among the methane bacteria, which may result from a peculiarity of the phosphoroclastic system of strain BERRE S. The enhanced activity of nucleotide mixtures and the effects of GTP, UTP etc. may be related to the crudity of the test system.

Relatively little of the energy and/or reducing power of the phosphoroclastic system is channelled into methane formation and it is reasonable to assume that the extent of channelling depends on the molar ratios of ATP, acetyl phosphate, co-enzyme A and their derivatives, which will normally assume steady states during the decomposition of pyruvate. It is difficult to draw precise conclusions about the influences of these factors added as single substances, when their addition in large amounts will drastically influence the steady state concentration of the others. It seems likely that the need for

large amounts of co-enzyme A reflected a necessity to unbalance a control system which normally limits drastically the amounts of methane formed.

There are reasons for regarding methane production, pyruvic phosphoroclastism, nitrogen fixation and non-haem-iron electron transport as biologically primitive reactions. Peck (1967) and Klein & Cronquist (1967) have proposed that both methane-producing bacteria and sulphate-reducing bacteria are present-day representatives of very primitive living things. The existence of a methane-producing system, biochemically similar to that in methane bacteria, in one of the sulphate-reducing bacteria, together with the detectable activity of comparable systems in some other bacteria capable of pyruvic phosphoroclastism, adds weight to this view. Methane formation in *Desulfovibrio* species and the other non-methane bacteria may be a 'vestigial' biochemical process of the kind proposed by Kelly (1968) for *Thiobacillus neapolitanus* and by Gottschalk (1968) for desulfovibrios and certain clostridia.

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