The Effect of Hydrogen on the Growth of Desulfovibrio vulgaris (Hildenborough) on Lactate

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Desulfovibrio vulgaris (Hildenborough) was grown on lactate with either a \( \text{N}_2/\text{CO}_2 \) or a \( \text{H}_2/\text{CO}_2 \) gas phase. \( \text{H}_2 \) increased the growth yield on lactate and had a sparing effect on lactate utilization, without altering the growth rate or hydrogenase level. Growth on acetate plus \( \text{CO}_2 \) with \( \text{H}_2 \) as sole energy source did not require an extensive adaptation period. Addition of lactate to cultures growing on acetate and \( \text{H}_2/\text{CO}_2 \) resulted in a switch from acetate to lactate utilization. In lactate-limited medium under \( \text{H}_2/\text{CO}_2 \) biphasic growth was observed. Lactate was oxidized first with production of acetate, followed by a second phase of growth on the acetate. Under this condition \( \text{H}_2 \) did not provide any supplementary energy during growth on lactate, as was evident from the ratio of lactate utilized to acetate produced.

INTRODUCTION

Sulphate-reducing bacteria (SRB) constitute an integral part of mixed microbial populations or consortia in sulphate-containing environments (Pfenning & Widdel, 1982; Hamilton, 1985). Their diverse metabolic capabilities (Peck & LeGall, 1982; Odom & Peck, 1984) allow them to carry out various interactions in different ecological niches such as marine and estuarine sediments, and biofilms. SRB are capable of growth on a wider range of substrates than had been previously appreciated (Pfennig et al., 1981), and are thus very flexible ecologically. The relative importance of various intermediates of the mineralization process for the growth of SRB has recently been receiving increased attention (Jørgensen, 1982). Acetate in particular has been shown to be an important substrate for sulphate reduction, accounting for between 50% and 65% of the electron donors in marine environments (Sørensen et al., 1981; Christensen, 1984). Many SRB capable of growth on acetate as sole source of carbon and energy with sulphate as terminal electron acceptor have been isolated by F. Widdel (Pfennig et al., 1981).

Desulfovibrio vulgaris, on the other hand, is only able to grow on organic compounds such as lactate, oxidizing them incompletely to acetate. Badzioch et al. (1978), however, isolated a Desulfovibrio species which utilized \( \text{H}_2 \) as the sole energy source and acetate and \( \text{CO}_2 \) as carbon sources. Approximately 70% of the cell carbon was derived from acetate and 30% from \( \text{CO}_2 \). It was believed at the time that this organism differed from the type strain \( D. vulgaris \) (Hildenborough) in this capacity to grow on \( \text{H}_2 \) in a minimal medium with acetate and \( \text{CO}_2 \). However, Brandis & Thauer (1981) were able to adapt \( D. vulgaris \) (Hildenborough) and other Desulfovibrio spp. to grow on \( \text{H}_2 \) as energy source.

\( \text{H}_2 \) is now believed to play a central role in the metabolism of Desulfovibrio, although the physiological function of \( \text{H}_2 \) and hydrogenase in the growth of SRB remains the subject of much discussion (Odom & Peck, 1984). Many Desulfovibrio spp. have the unique ability not only to oxidize \( \text{H}_2 \) (Brandis & Thauer, 1981; Nethe-Jäenchen & Thauer, 1984) but also to produce it,

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Abbreviation: SRB, sulphate-reducing bacteria.

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even in the presence of sulphate (Tsuij & Yagi, 1980; Traore et al., 1981). The possible physiological significance of this phenomenon has been discussed by several workers (Hatchikian et al., 1976; Tsuij & Yagi, 1980; Odom & Peck, 1981, 1984; Lupton et al., 1984). One hypothesis is the obligate H₂-cycling mechanism linked to chemiosmotic energy metabolism (Odom & Peck, 1981). Electrons from lactate oxidation are transferred to protons, producing H₂ by the cytoplasmic hydrogenase. This H₂ then diffuses across the cell membrane where it is oxidized by the periplasmic hydrogenase, creating a proton gradient across the membrane and thus allowing energy transduction. Another model by Lupton et al. (1984) envisages the production of internal H₂ by cytoplasmic hydrogenase as regulating the redox state of electron carriers and so preventing the loss of energy by oxidizing H₂ in the periplasm.

In the investigation described here, the effect of H₂ on growth, lactate oxidation and hydrogenase levels in _D. vulgaris_ was studied. The capability of H₂-acetate-grown cultures to utilize lactate was also investigated.

**METHODS**

*Organism and media. D. vulgaris* (Hildenborough) NCIB 8303 was obtained from the National Collections of Industrial and Marine Bacteria Ltd, Aberdeen, UK. The bacteria were maintained in a medium containing (g l⁻¹): Na₂SO₄, 3-0; KH₂PO₄, 0-2; NH₄Cl, 0-25; NaCl, 1-0; MgCl₂, 6H₂O, 0-4; KCl, 0-5; CaCl₂, 2H₂O, 0-15; resazurin, 0-001; sodium lactate (70% w/w), 5 ml. The medium was boiled, cooled under O₂-free N₂ and 50 ml volumes were dispensed into 100 ml Wheaton bottles (Supelco).

The bottles were stoppered with black butyl rubber stoppers and clamped with aluminium caps (Bellco). A solution of Na₂S, 9H₂O (1% w/v) was injected into each bottle to give a final concentration of 0-01 g l⁻¹. The culture bottles were pressurized to 0-5 bar with N₂ and autoclaved for 20 min at 121 °C. After autoclaving, 1 ml 100% sterile anoxic trace element solution (Widdel, 1980) was added and the pH adjusted to 6-8 with 0-3 ml 8-4% (w/v) NaHCO₃. The medium was inoculated with a 2% (v/v) inoculum and incubated at 35 °C. The culture was transferred into fresh medium every second day.

The growth medium contained the following (g l⁻¹): (NH₄)₂SO₄, 6-6; NaCl, 1-8; KH₂PO₄, 0-9; MgCl₂, 6H₂O, 0-36; CaCl₂, 2H₂O, 0-26; resazurin, 0-001; sodium lactate (70% w/w), 5 ml or sodium acetate, 2-5; trace mineral solution (Brandis & Thauer, 1981), 12-5 ml. The trace mineral solution was autoclaved separately and the pH of the medium adjusted to 6-8 with sterile 10% (w/v) Na₂CO₃.

**pH-stat batch culture.** The apparatus is shown in Fig. 1. Each vessel was autoclaved aerobically and then connected up with butyl rubber tubing (Esco Rubbers). The gas mixture (I) (80:20, v/v, N₂/CO₂) was passed over copper turnings which were heated at 350 °C to remove traces of O₂ and through a 11 bottle (II) containing sterile distilled water and resazurin (0-001 g l⁻¹). It was then bubbled through a 51 bottle (III) containing sterile medium before entering the culture vessel (IV), which contained 400 ml growth medium. After equilibration with N₂/CO₂ or H₂/CO₂ (flow rate 250 ml min⁻¹), 10 ml 5% (w/v) Na₂S, 9H₂O was added to the 11 bottle (II) and within 20 min the medium was reduced as evidenced by the disappearance of the blue colouration of the resazurin. The pH in the culture vessel was maintained at 6-8 by the automatic addition of 10% (v/v) H₂SO₄ using a pH controller (LH Engineering). A butyl rubber tubing connection was made between the bottle containing acid and the culture vessel (IV) so as to compensate for the negative pressure that would be formed due to the automatic removal of acid from the bottle. The samples for the measurement of growth, substrate utilization and product formation were withdrawn from the culture vessel using the sampling port (V).

**Hydrogenase.** The enzyme was assayed as H₂-consuming activity by following spectrophotometrically the reduction of methyl viologen at 578 nm (Pankhania et al., 1986a), and as H₂-producing activity by an electrochemical method using a H₂-electrode (Hansatech) (Gow et al., 1986). Hydrogenase activity was sensitive to inactivation by O₂ if the cells were grown under H₂/CO₂, whereas under N₂/CO₂ the enzyme was extremely stable (I. P. Pankhania, unpublished results). For this reason the cells grown under a H₂/CO₂ gas phase were collected anaerobically (Pankhania et al., 1986b). Cultures were centrifuged at 10000 g for 20 min and the pellets were resuspended in 50 mM-Tris/HCl buffer, pH 7-5, containing 50 mM-NaCl and 1 mM-dithiothreitol. Cells were broken by passage through a French pressure cell, centrifuged at 10000 g for 20 min, and the supernatants were examined for the hydrogenase activities in the absence or presence of CO as described by Gow et al. (1986).
Growth of Desulfovibrio vulgaris on lactate

**Measurement of sulphate, lactate and acetate.** Sulphate was assayed turbidimetrically (Taras et al., 1971). Lactate and acetate were measured by gas chromatography (Pye-Unicam PU4500) on a GP 15% SP-1220/1% H₂PO₄ on 100–120 Chromosorb WAW (Supelco) column (1.82 m × 4 mm i.d.) with a flame ionization detector. The carrier gas was N₂ at a flow rate of 35 ml min⁻¹. The flow rates of H₂ and air were 45 ml min⁻¹ and 450 ml min⁻¹ respectively. The temperatures of the detector and injector were 170 °C and that of the column was 115 °C. Culture (5 ml) was centrifuged at 13000 g in a Micro Centaur (MSE) centrifuge for 10 min and the supernatant was used for the analyses. For acetate, 500 µl supernatant was acidified with 50 µl concentrated HCl and a 2 µl sample was injected in the chromatograph. Lactate was first methylated as described in the Supplement to Supelco Bulletin 748F and a 2 µl sample was used for analysis. Appropriate standards were run to calculate the concentrations of acetate and lactate in the samples.

**RESULTS**

**Effect of H₂ on growth on lactate**

Growth of SRB is generally poor in closed bottles because of the toxic effects of the H₂S produced (Postgate, 1984; Klemps et al., 1985). Additionally, even if the medium is well chelated the accumulation of sulphide can cause precipitation of iron, which makes it difficult to measure growth by following the increase in optical density. A further problem in our experiments was that during growth of *D. vulgaris* under N₂/CO₂ the pH shifted from 6.8 to 7.2, whereas under H₂/CO₂ the increase was up to pH 7.8, which resulted in lysis of the cells (data not shown). Therefore, the batch culture with a pH controller was set up in which at the constant pH of 6.8, sulphide was removed from the culture as H₂S in the gas stream (Fig. 1).

Growth of *D. vulgaris* on lactate under N₂/CO₂ or H₂/CO₂ was followed by measuring increase in optical density, lactate utilization, acetate production and hydrogenase activity (data not shown). Growth stopped upon exhaustion of lactate. The periplasmic hydrogenase activity increased with time and followed growth. No increase in the hydrogenase activity was observed when the enzyme was assayed in the presence of Triton X-100. Odom & Peck (1981) have reported an increase in the enzyme activity in the presence of Triton X-100 which has been thought to be due to the presence of a second cytoplasmic hydrogenase.
Fig. 2. Growth (○) of D. vulgaris on lactate under N₂/CO₂ followed by H₂/CO₂ (the change in gas phase is indicated by the dashed line). Lactate (△) and acetate (▴) were measured by gas chromatography as described in Methods.

Fig. 3. Growth (○) of D. vulgaris on acetate under H₂/CO₂. The arrow indicates the time (9 h) when 25 mM-lactate was added to the growth vessel. Lactate (△), acetate (▴) and sulphate (●) were measured as described in Methods.

Table 1. Growth parameters of D. vulgaris on lactate under N₂/CO₂ or H₂/CO₂ gas phases

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>Y (g l⁻¹)</th>
<th>t₀ (h)</th>
<th>Lactate/acetate</th>
<th>Yₑactate (g dry wt cells mol⁻¹)</th>
<th>Hydrogenase* [U (g dry wt)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂/CO₂</td>
<td>0.255</td>
<td>3.7</td>
<td>1.17</td>
<td>5.95</td>
<td>10.3</td>
</tr>
<tr>
<td>H₂/CO₂</td>
<td>0.43</td>
<td>3.8</td>
<td>1.70</td>
<td>8.75</td>
<td>9.56</td>
</tr>
</tbody>
</table>

* U = mmol H₂ produced or consumed min⁻¹.

When the gas phase was changed from N₂/CO₂ to H₂/CO₂ a short lag of about 1 h was observed before there were further increases in cell mass (Fig. 2) and hydrogenase activities (data not shown). The growth parameters under N₂/CO₂ and H₂/CO₂ gas phases are summarized in Table 1. H₂ had a pronounced effect on growth of D. vulgaris on lactate. In the presence of H₂, there were increases in growth yield and in the ratio of lactate consumed to acetate produced. However, there was no significant effect on the growth rate or the hydrogenase activity, suggesting that the periplasmic hydrogenase may be a constitutive enzyme. Hydrogenase activity was therefore measured in crude extracts of D. vulgaris grown with lactate under N₂/CO₂ or H₂/CO₂, and with acetate under H₂/CO₂. The activities were respectively, 5.7, 5.2 and 3.4 μmol H₂ produced min⁻¹ (mg protein)⁻¹. When the enzyme was measured in the presence of CO₂ however, cells grown on lactate under N₂/CO₂ retained nearly 18% of the original activity (1.0 μmol H₂ min⁻¹ mg⁻¹). This indicates that under such growth conditions there may be more membrane-bound hydrogenase (Gow et al., 1986). Comparable figures for lactate and acetate under H₂/CO₂ were respectively, 3.8% (0.2 μmol H₂ min⁻¹ mg⁻¹) and 5.9% (0.2 μmol H₂ min⁻¹ mg⁻¹).
Growth of *Desulfovibrio vulgaris* on lactate

It was not known whether cultures adapted to grow on \( \text{H}_2/\text{CO}_2 \) and acetate would utilize lactate. The data presented above show that *D. vulgaris* did not use acetate as a carbon source when growing on lactate with either \( \text{N}_2/\text{CO}_2 \) or \( \text{H}_2/\text{CO}_2 \) as gas phase. However, the addition of lactate to cultures growing with \( \text{H}_2 \) as energy source resulted in a switch from acetate to lactate utilization, which was evident from the further production of acetate (Fig. 3).

Growth with \( \text{H}_2 \) as the sole energy source

*D. vulgaris* (Hildenborough) was originally thought to differ from *D. vulgaris* strains Marburg and Madison in that it could not be grown with \( \text{H}_2 \) as sole energy source (Badziong et al., 1978). It was later demonstrated that the ability to grow on \( \text{H}_2 \) was common to other species (Brandis & Thauer, 1981); *D. vulgaris* (Hildenborough), however, required an elaborate adaptation period prior to growth on \( \text{H}_2 \). In our studies no such adaptation period was required (Pankhania et al., 1986b) although a lag period of about 18 h was observed when a lactate-grown culture was subcultured into acetate medium under \( \text{H}_2/\text{CO}_2 \). After four subcultures growth and acetate utilization were measured (data not shown). The growth rate was similar to that observed with lactate.

Growth of *D. vulgaris* in lactate-limited medium

Growth of *D. vulgaris* in medium containing 9 mM-lactate was followed under a gas phase of \( \text{N}_2/\text{CO}_2 \). The growth rate was low and growth stopped when lactate was completely utilized (Fig. 4), the acetate produced from lactate not being utilized further. However, when the gas phase was changed to \( \text{H}_2/\text{CO}_2 \), growth was much faster, after a lag phase of approximately 2 h. When the above experiment was carried out entirely under \( \text{H}_2/\text{CO}_2 \), a biphasic growth pattern was observed, lactate first being oxidized to acetate, followed immediately by growth on acetate after exhaustion of the lactate (Fig. 5). A surprising observation was that the ratio of lactate consumed to acetate formed was nearly 1 rather than 2 as found with excess lactate (Table 1). These data suggest that \( \text{H}_2 \) may not serve as an energy source in the presence of limiting lactate.
DISCUSSION

One of the aims of this work was to look for evidence for or against the H₂-cycling mechanism as proposed by Odom & Peck (1981). A major problem for this hypothesis is that the standard redox potentials for the two half reactions lactate → pyruvate + 2H⁺ + 2e⁻ (E°' = −197 mV) and 2H⁺ + 2e⁻ → H₂ (E°' = −420 mV) are such that H₂ production from the oxidation of lactate is endergonic. Because of the unfavourable position of the equilibrium of the reaction therefore, the oxidation of lactate can only proceed at very low H₂ concentrations and/or high lactate:pyruvate ratios. Even with a lactate:pyruvate ratio of 100:1, this would require a partial pressure of H₂ less than 10⁻⁵ atm. According to the H₂-cycling hypothesis therefore, oxidation and growth on lactate should be inhibited by H₂ partial pressures in excess of 10⁻⁵ atm. Data obtained in this study, however, suggest that H₂ did not inhibit lactate oxidation. Instead it had a sparing effect on lactate utilization and increased the growth yield on lactate. Lupton et al. (1984) also observed no inhibition of growth or substrate consumption when D. vulgaris (Madison) was cultured on lactate in the presence of H₂. We have additionally shown that D. vulgaris grown on lactate in a sulphate- (10 mM) limited chemostat does not suffer wash-out at low dilution rate under a H₂/CO₂ gas phase (I. P. Pankhania, unpublished results). Similar results using sulphate-limited chemostats were obtained by Nethe-Jaenchen & Thauer (1984) with D. vulgaris (Marburg) and by Cypionka & Pfennig (1986) with Desulfotomaculum orientis.

Other evidence against the H₂-cycling hypothesis comes from the lack of inhibition of synthesis or activity of lactate dehydrogenase by H₂ (Hamilton, 1982) and the production of H₂ during growth on lactate plus sulphate (Tsujii & Yagi, 1980; Traore et al., 1981). Lupton et al. (1984) have proposed a trace H₂ transformation model for the physiological function of H₂ metabolism during growth of D. vulgaris on organic electron donors. In this model, hydrogenases function in regulating electron flow and preventing energy loss by producing and consuming H₂ respectively. An alternative explanation would be that the energetically unfavourable oxidation of lactate is driven by energy-dependent reversed electron transport in line with the model suggested by Thauer & Morris (1984).

Another requirement for the mechanism of H₂-cycling is the presence of two hydrogenases, one in the periplasm, functioning as a H₂-consuming hydrogenase, and another in the cytoplasm, functioning as a H₂-producing hydrogenase (Odom & Peck, 1984). Our results suggest that two hydrogenases are present in D. vulgaris. The second, membrane-bound hydrogenase can be detected in the presence of CO. The membrane-bound enzyme differs from periplasmic hydrogenase in a number of properties: (a) R₅ values are 0-12 and 0-45 for membrane-bound and periplasmic hydrogenase respectively, (b) membrane-bound hydrogenase does not cross react with antisera raised against periplasmic hydrogenase but it is digested by pancreatin, and (c) the membrane-bound enzyme is not inhibited by CO (Gow et al., 1986). Evidence for two hydrogenases, one a CO-sensitive enzyme and the other CO-stimulated, has also been reported in D. vulgaris (Madison) by Lupton et al. (1984). Although we have found evidence for a second hydrogenase, we still do not know the physiological role of this enzyme.

The highest activity of the membrane-bound enzyme was found in the cells grown on lactate under N₂/CO₂.

In lactate-limited growth conditions under N₂/CO₂, lactate was oxidized to acetate and no further oxidation of acetate occurred. It is well established that D. vulgaris lacks a complete tricarboxylic acid cycle (Lewis & Miller, 1977). However, under a H₂/CO₂ gas phase, biphasic growth occurred. Firstly lactate was oxidized, followed by acetate utilization for the anabolic pathways (Badziong et al., 1978), using H₂ as sole energy source. In lactate-limited conditions H₂ did not provide any supplementary energy, as was evident from the lactate/acetate ratio. Similar results were obtained by Khosrovi et al. (1971); as yet we are unable to explain the above observation physiologically. Another important point which must be mentioned here is that Khosrovi & Miller (1975) reported specific growth yields of 9.2 and 25.8 g (mol lactate⁻¹ for D. vulgaris under Ar/CO₂ (95:5, v/v) and H₂/CO₂ (95:5, v/v) respectively. However, the yields reported in the present paper are much lower and H₂ increased the yield by only 47%. The apparent 180% increase in yield reported by Khosrovi & Miller (1975) might be explained on the basis that the culture was in fact capable of biphasic growth under H₂/CO₂, growing on lactate first then switching to a second phase of growth on acetate.
In this study we have shown that if D. vulgaris is given a choice between lactate and acetate it will preferentially utilize lactate irrespective of the presence or absence of H₂. Also the capacity of D. vulgaris to grow biphasically on lactate and acetate may have a significant ecological implication.

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REFERENCES


