Keto Acid Metabolism in *Desulfovibrio*

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SUMMARY

Four strains of *Desulfovibrio* each excreted pyruvate to a constant level during growth; it was re-absorbed when the substrate (lactate) was exhausted. Malate, succinate, fumarate and malonate also accumulated during growth.

One of the strains (Hildenborough) excreted α -ketoglutarate as well as pyruvate when incubated in nitrogen-free medium; the former was re-absorbed on addition of NH_4Cl . In a low-lactate nitrogen-free medium, strain Hildenborough rapidly re-absorbed the pyruvate initially excreted, but did not re-absorb the α -ketoglutarate. Arsenite (1 mM) prevented the accumulation of α -ketoglutarate; 1 mM-malonate did not affect the accumulation of keto acids.

Isocitrate dehydrogenase activity (NAD-specific) in all strains was lower than NADP-specific glutamate dehydrogenase activity. α -Ketoglutarate dehydrogenase could not be detected in any strain. NADPH oxidase activity was demonstrated.

This and previous work indicate that a tricarboxylic acid pathway from citrate to α -ketoglutarate exists in *Desulfovibrio* spp., and that succinate can be synthesized via malate and fumarate; however, an intact tricarboxylic acid cycle is evidently not present. The findings are compared with observations on biosynthetic pathways in clostridia, obligate lithotrophs, phototrophs, and methylotrophs, and various facultative bacteria.

INTRODUCTION

Desulfovibrio species utilize sulphate as terminal electron acceptor in anaerobic respiration, and simple organic substances such as lactate, malate or pyruvate as carbon sources. The tricarboxylic acid (TCA) cycle has not been shown to occur in *Desulfovibrio*, although several reactions of the cycle have been demonstrated (Grossman & Postgate, 1955; Miller & Wakerley, 1966; Gottschalk, 1968; Miller *et al.* 1970; Hatchikian & Le Gall, 1970). An important reaction for the cycle, which has not been conclusively shown, is that catalysed by α -ketoglutarate dehydrogenase.

There have been demonstrations of extracellular accumulation of α -ketoglutarate by *Pseudomonas aeruginosa* (von Tigerstrom & Campbell, 1966) and *Brevibacterium flavum* (Shiio, Otsuka & Takahashi, 1961) during metabolism of substrate in the absence of ammonium ions. In the work reported here we observed the accumulation of α -ketoglutarate and pyruvate by physiologically active *Desulfovibrio vulgaris* in a medium lacking combined nitrogen. The accumulation of other organic acids during the growth of *Desulfovibrio* spp. was also examined.

METHODS

Organisms and culture methods. *Desulfovibrio vulgaris* strains Hildenborough (NCIB8303) and Woolwich (NCIB8457), and *D. desulfuricans* strains Teddington R (NCIB8312) and Byron (NCIB8458) were grown in the chemically-defined liquid medium of Macpherson &

Miller (1963), which contains lactate as sole carbon source. Cultures (200 ml) in cotton-wool-plugged Pyrex conical flasks were incubated at 30 °C in McIntosh & Fildes anaerobic jars under H₂. Growth was measured by determining extinction at 600 nm.

Cultures for nitrogen starvation studies were harvested aseptically, before growth was complete, by centrifuging at 15000 *g* for 5 min. The organisms were then washed in sterile tris-HCl buffer (0.1 M, pH 7.2), recentrifuged, and suspended in 200 ml sterile nitrogen-free (non-growth) medium which differed from the original medium in containing 5 or 50 mM-sodium lactate but no NH₄Cl. After incubation as before, cultures were centrifuged at 20000 *g* for 15 min to remove the bacteria, the supernatants then being adjusted to pH 7.4.

Chromatography. The g.l.c. technique was a modification of the method of Harman & Doelle (1969). Methyl esters of the TCA cycle intermediates, along with those of lactate, pyruvate and malonate, were prepared by the method of Harman & Doelle (1969). Supernatant samples (50 ml) were dried in a rotary vacuum evaporator at 40 °C; the residues were esterified, dissolved in chloroform and 5 µl injected onto a column of 10 % Reoplex on acid-washed Chromosorb W (60 to 80 mesh). Columns, 1 m × 6.3 mm (obtained from Phase Separations Ltd, Queensferry, Chester) were used on a Pye 104 gas chromatograph using a temperature programme from 50 to 190 °C at 6 °C/min and a final holding period of 10 min.

Quantitative estimation of organic acids. Commercial enzymes for use in the assays were obtained from Sigma.

Lactic acid was measured enzymically by an adaptation of the method of Rosenberg & Rush (1966). Glycine-semicarbazide buffer was prepared daily: 1.5 g glycine + 2.2 g semicarbazide in 80 ml distilled water were adjusted to pH 10 with 2 M-NaOH and made up to 100 ml. The assay mixture contained (ml): buffer, 2.8; NAD⁺ (5 mg/ml), 0.6; sample, 0.2. The extinction at 340 nm was read (*E*₁) before the addition of 0.4 ml lactate dehydrogenase (beef heart; 20 units/ml). The mixture was then incubated at 40 °C for 2 h and the *E*₃₄₀ again read (*E*₂). A blank containing distilled water instead of the sample was set up at the same time and readings taken (*B*₁ and *B*₂). The net change in extinction, Δ*E*, as a result of the NADH formed by the oxidation of lactate to pyruvate was given by

$$\Delta E = (E_2 - 0.9E_1) - (B_2 - 0.9B_1).$$

Pyruvic and α-ketoglutaric acids were measured both chemically and enzymically. By the chemical method of Goodwin & Williams (1952), both acids were measured in one assay. Pyruvate was determined enzymically by the method of Rosenberg & Rush (1966). α-Ketoglutarate was measured using glutamate dehydrogenase. The assay mixture contained (ml): tris-HCl buffer (0.05 M, pH 7.4), 1.7; NADH (2 mg/ml), 0.1; NH₄Cl (0.1 M), 0.1; glutamate dehydrogenase (bovine liver; 25 units/ml), 0.1; sample, 1.0. The reaction was followed at 340 nm and the initial rate of the reaction compared with a calibration curve. All extinctions were determined on a Beckman DB spectrophotometer; enzyme units are expressed as that amount which catalyses the removal of 1 µmol substrate in 1 min at 30 °C.

Preparation of extracts. Bacteria were grown in 4 l batches and harvested using a continuous flow head attachment on an MSE 18 centrifuge (flow rate 30 to 50 ml/min). The bacteria were then resuspended in tris-HCl buffer (0.05 M, pH 7.4), the suspension placed in a French press and the bacteria disrupted at 20000 lb/in² (13.7 MPa). The extract was centrifuged at 15000 *g* for 30 min, the supernatant decanted in 1 ml portions and stored below 0 °C.

Assay of isocitrate dehydrogenase (EC. 1.1.1.41). As extracts of the bacteria contain a NADH oxidase (Germano & Anderson, 1968), it was impossible to measure the enzyme activity by following Δ*E*₃₄₀ because of NAD formation. Instead, the enzyme was assayed

by examining the effect of isocitrate on the oxidation of NADH in the presence of NAD. The assay mixture contained (per 3.0 ml): isocitrate, 10 μ mol; NAD, 0.3 μ mol; NADH, 0.26 μ mol; MnSO_4 , 5 μ mol; tris-HCl buffer pH 7.4, 125 μ mol; cell-free extract, 0.1 ml. NADH oxidation was measured using the above mixture without isocitrate; isocitrate dehydrogenase activity was the difference between the two rates.

Assay of aconitate hydratase (EC. 4.2.1.3). This was measured by determining the amount of isocitrate formed from *cis*-aconitate using isocitrate dehydrogenase (NADP-specific). During the course of these experiments NADPH oxidase activity was observed, which interfered with the assay. A similar assay was therefore used to that for isocitrate dehydrogenase above. The mixture contained (per 3.0 ml): tris-HCl buffer pH 7.4, 125 μ mol; aconitate, 15 μ mol; NADP, 0.3 μ mol; NADPH, 0.1 μ mol; MnSO_4 , 5 μ mol; isocitrate dehydrogenase (NADP-specific, pig heart), 10 units; cell-free extract, 0.1 ml. The reference cuvette contained the same except for the NADPH and *cis*-aconitate, which were used to start the reaction. The NADPH oxidation rate was measured in the absence of aconitate.

Assay of α -ketoglutarate dehydrogenase. The NADH oxidase also affected this assay, so a similar method to that for isocitrate dehydrogenase was used. The mixture contained (per 3.0 ml): tris-HCl buffer pH 8.2, 125 μ mol; MgCl_2 , 5 μ mol; NAD, 0.3 μ mol; NADH, 0.26 μ mol; cysteine-HCl, 3 μ mol; thiamin pyrophosphate, 0.2 μ mol; coenzyme A, 0.1 μ mol; α -ketoglutarate, 10 μ mol; cell-free extract, 0.1 ml. The reference cuvette contained all these components except NADH and α -ketoglutarate, which were used to start the reaction. The rate of NADH oxidation was measured in the absence of α -ketoglutarate.

Another assay was also used, based on the colorimetric determination of ferrocyanide produced by the oxidative decarboxylation of α -ketoglutarate with ferricyanide as electron acceptor (Hager, 1953).

Assay of glutamate dehydrogenase (EC. 1.4.1.4). This enzyme was assayed spectrophotometrically by following the oxidation of NADPH in the presence of α -ketoglutarate and NH_4^+ at 340 nm. The mixture contained (per 3.0 ml): tris-HCl buffer pH 7.4, 130 μ mol; α -ketoglutarate, 10 μ mol; NH_4Cl , 10 μ mol; NADPH, 0.3 μ mol; cell-free extract, 0.1 ml. NADPH oxidation was measured in the above mixture without α -ketoglutarate.

RESULTS

Keto acid accumulation

In all the strains examined, pyruvate was the only keto acid to accumulate during growth in the complete medium of Macpherson & Miller (1963). Pyruvate formed rapidly and thereafter remained at a constant concentration (Fig. 1). In medium containing 5 mM-lactate (carbon-limiting) the pyruvate disappeared as the lactate was used up (Fig. 2).

There was a similar excretion of pyruvate by all strains in 100 mM-lactate nitrogen-free medium after transfer from full growth medium; strain Hildenborough also excreted α -ketoglutarate. In a 5 mM-lactate nitrogen-free medium the pyruvate initially excreted by strain Hildenborough was subsequently utilized along with lactate, but the α -ketoglutarate was not utilized (Fig. 3). The other strains did not excrete α -ketoglutarate in the low-lactate nitrogen-free medium, and when α -ketoglutarate was added to this medium it was not utilized. Figure 4 shows the results obtained with strain Teddington R; strains Woolwich and Byron gave closely similar results.

Clearly, ammonium ions suppressed α -ketoglutarate excretion into full growth medium by strain Hildenborough, while their addition to a suspension of this strain in nitrogen-free medium rapidly stopped the accumulation of the keto acid and allowed it to be utilized

Fig. 1

Fig. 2

Fig. 1. Concentration of lactate (Δ) and pyruvate ($\circ$) in the medium, during growth ($\bullet$) of *D. vulgaris* strain Hildenborough in Macpherson & Miller's (1963) medium.

Fig. 2. Concentration of lactate (Δ) and pyruvate ($\circ$) in the medium, during growth ($\bullet$) of strain Hildenborough in a 5 mM-lactate modification of Macpherson & Miller's (1963) medium.

Fig. 3

Fig. 4

Fig. 3. Concentration of lactate (Δ), pyruvate ($\circ$) and α -ketoglutarate ($\bullet$), in the cell-free supernatant from a suspension of strain Hildenborough in a 5 mM-lactate nitrogen-free medium.

Fig. 4. Concentration of lactate (Δ), pyruvate ($\circ$) and α -ketoglutarate ($\bullet$), in the cell-free supernatant from a suspension of *D. desulfuricans* strain Teddington R in 5 mM-lactate nitrogen-free medium with 30 μ M- α -ketoglutarate added.

(Fig. 5). A similar uptake of α -ketoglutarate by the three other strains was observed on addition of ammonium ions to the nitrogen-free medium with added α -ketoglutarate, as shown for strain Teddington R in Fig. 5.

Malonate (1 mM), an inhibitor of succinate dehydrogenase, had no effect on the accumulation of keto acids, while arsenite (1 mM), an inhibitor of pyruvate and α -ketoglutarate dehydrogenases, prevented the accumulation of α -ketoglutarate, as also observed by Neumann (1967) with strain Hildenborough.

Accumulation of other acids

Gas-liquid chromatography of the supernatant medium after growth of strains Hildenborough and Teddington R showed that both strains excreted malate, fumarate, succinate and malonate during growth. Strain Hildenborough also excreted an acid which though not positively identified, had a similar retention time to butyrate.

Fig. 5. Uptake of α -ketoglutarate by strains Hildenborough and Teddington R suspended in nitrogen-free medium, after addition (arrow) of 10 mM- NH_4Cl . $\circ$, Strain Hildenborough, with no added α -ketoglutarate; $\bullet$, strain Teddington R, with 100 μM - α -ketoglutarate added.

Table 1. Levels of certain enzymes in four strains of *Desulfovibrio*

For details of assay conditions, see Methods.

	Specific activity (units/g protein)			
	<i>D. vulgaris</i>		<i>D. desulfuricans</i>	
	Strain Hildenborough	Strain Woolwich	Strain Teddington R	Strain Byron
Aconitate hydratase	3.4	3.3	3.7	4.0
Isocitrate dehydrogenase	7.2	6.6	7.5	7.1
Glutamate dehydrogenase	41.0	38.0	38.0	40.0
NADH oxidation	10.0	9.1	9.6	8.6
NADPH oxidation	1.1	0.9	1.5	1.4

In a parallel experiment, these two strains were grown in full medium as above, centrifuged before growth was complete, and resuspended in fresh growth medium to which 1 mM-arsenite or 1 mM-malonate had been added. Gas-liquid chromatography of the resulting culture supernatant gave very similar results. Since arsenite inhibits α -ketoglutarate synthesis but not that of malate, fumarate or succinate, α -ketoglutarate cannot be a precursor of these acids.

Enzyme levels

The enzymes of α -ketoglutarate metabolism were estimated in all four strains (Table 1). Formation of α -ketoglutarate by isocitrate dehydrogenase was found to be low compared with its conversion to glutamate. The isocitrate dehydrogenases were shown to be NAD-specific; the glutamate dehydrogenases were NADP-specific. No activity of α -ketoglutarate dehydrogenase was detected by two different assay methods.

DISCUSSION

The accumulation of pyruvate by *Desulfovibrio* in full growth medium and nitrogen-free medium must be due to the initial conversion of lactate to pyruvate being faster than the utilization of pyruvate. Some control, however, must be exerted so that the extracellular pyruvate concentration remains unchanged. Why α -ketoglutarate is excreted into the

nitrogen-free medium only by *D. vulgaris*, strain Hildenborough, of the four strains examined is not clear. Macpherson & Miller (1963) showed that the Hildenborough strain grew poorly on glutamate or glutamine as sole carbon source, but they did not grow the organism on α -ketoglutarate. As α -ketoglutarate is not used in nitrogen-free medium when other carbon sources are exhausted, the bacteria are probably unable to oxidize it. Since the keto acid disappears rapidly when ammonium ions are added to the medium, presumably to glutamate, a permease must be present.

Although α -ketoglutarate dehydrogenase activity was not detected in cell-free extracts, this does not prove conclusively that the enzyme is absent. As isocitrate dehydrogenase activity is much lower than that of glutamate dehydrogenase, any α -ketoglutarate formed would immediately be used for glutamate synthesis, rather than oxidized with a TCA cycle.

The accumulation of α -ketoglutarate only by strain Hildenborough is possibly due to the systems of α -ketoglutarate production in the other three strains being subject to end-product repression.

Gottschalk (1968) has shown that α -ketoglutarate is not formed via succinate in sulphate-reducing bacteria; hence the effect of arsenite in inhibiting α -ketoglutarate formation must be on pyruvate dehydrogenase. This means that malate, succinate and fumarate could not be formed via the TCA cycle in the conventional direction. A possible sequence is malate $\rightarrow$ fumarate $\rightarrow$ succinate, with malate arising from pyruvate by the action of the malic enzyme. Malonate has no effect on the accumulation of these acids. Some support for this sequence comes from the work of Barton & Peck (1971) and Barton, Le Gall & Peck (1973), who used particulate preparations of *Desulfovibrio gigas*.

These findings all point to the existence of an incomplete TCA cycle in *Desulfovibrio*, having only a biosynthetic function. This is similar to what is currently understood of the functions of the cycle in anaerobic bacteria. Incomplete TCA cycles in clostridia (Stern & Bambers, 1966), in *Desulfovibrio* and in anaerobically grown *Escherichia coli* indicate that anaerobes use only parts of the cycle as biosynthetic pathways. In *E. coli*, α -ketoglutarate dehydrogenase is repressed on transfer to anaerobic conditions (Amarasingham & Davis, 1965) and a reductive pathway to succinate becomes operative (Hager & Kornberg, 1961). The cycle then operates as two separate biosynthetic pathways.

Obligate lithotrophs (Smith, London & Stanier, 1967; Johnson & Abraham, 1969; Peeters, Liu & Aleem, 1970; Tabita & Lundgren, 1971), obligate phototrophs (Fuller *et al.* 1961; Smith *et al.* 1967; Pearce, Leach & Carr, 1969) and obligate methylotrophs (Colby & Zatman, 1972; Dahl, Mehta & Hoare, 1972) have also been shown to lack a complete cycle or to possess one whose enzymes are very much lower in activity than those in the facultative members of each group.

The lack of α -ketoglutarate dehydrogenase and NADH oxidase in strict autotrophs was put forward as a biochemical explanation of their inability to grow heterotrophically by Smith *et al.* (1967). NADH oxidase has, however, been found in autotrophs (Aleem, 1968; Trudinger & Kelly, 1968), though the presence of an incomplete cycle remains a possible explanation.

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REFERENCES

- ALEEM, M. I. H. (1968). Mechanism of oxidative phosphorylation in the chemoautotroph *Nitrobacter agilis*. *Biochimica et biophysica acta* **162**, 338–347.

- AMARASINGHAM, C. R. & DAVIS, B. D. (1965). Regulation of α -ketoglutarate dehydrogenase formation in *Escherichia coli*. *Journal of Biological Chemistry* **240**, 3664–3668.
- BARTON, L. L., LE GALL, J. & PECK, H. D. (1973). In *Horizons of Bioenergetics*, p. 33. Edited by A. San Pietro and H. Gest. New York: Academic Press.
- BARTON, L. L. & PECK, H. D. (1971). Phosphorylation coupled to electron transfer between lactate and fumarate in cell-free extracts of the sulfate-reducing anaerobe, *Desulfovibrio gigas*. *Bacteriological Proceedings*, 149.
- COLBY, J. & ZATMAN, L. J. (1972). Hexose phosphate synthase and tricarboxylic acid cycle enzymes in bacterium 4B6, an obligate methylotroph. *Biochemical Journal* **128**, 1373–1376.
- DAHL, J. S., MEHTA, R. J. & HOARE, D. S. (1972). New obligate methylotroph. *Journal of Bacteriology* **109**, 916–921.
- FULLER, R. C., SMILLIE, R. M., SISLER, E. C. & KORNBERG, H. L. (1961). Carbon metabolism in *Chromatium*. *Journal of Biological Chemistry* **236**, 2140–2149.
- GERMANO, G. J. & ANDERSON, K. E. (1968). Purification and properties of L-alanine dehydrogenase from *Desulfovibrio desulfuricans*. *Journal of Bacteriology* **96**, 55–60.
- GOODWIN, T. W. & WILLIAMS, G. R. (1952). Studies on vitamin A. XVIII. The effect of vitamin A deficiency on the pyruvate and α -ketoglutarate levels of rat blood. *Biochemical Journal* **51**, 708–714.
- GOTTSCHALK, G. (1968). The stereospecificity of the citrate synthase in sulfate-reducing and photosynthetic bacteria. *European Journal of Biochemistry* **5**, 346–351.
- GROSSMAN, J. P. & POSTGATE, J. R. (1955). The metabolism of malate and certain other compounds by *Desulphovibrio desulfuricans*. *Journal of General Microbiology* **12**, 429–445.
- HAGER, L. P. (1953). Ph.D. thesis, University of Illinois.
- HAGER, L. P. & KORNBERG, H. L. (1961). On the mechanism of α -oxoglutarate oxidation in *Escherichia coli*. *Biochemical Journal* **78**, 194–198.
- HARMAN, M. A. & DOELLE, H. W. (1969). Gas chromatographic separation and determination of micro-quantities of the esters of the tricarboxylic acid cycle acids and related compounds. *Journal of Chromatography* **42**, 157–169.
- HATCHIKIAN, E. C. & LE GALL, J. (1970). Étude du métabolisme des acides dicarboxyliques et du pyruvate chez les bactéries sulfato-réductrices. *Annales de l'Institut Pasteur* **118**, 125–142.
- JOHNSON, E. J. & ABRAHAM, S. (1969). Assimilation and metabolism of exogenous organic compounds by the strict autotrophs *Thiobacillus thioarvus* and *Thiobacillus neapolitanus*. *Journal of Bacteriology* **97**, 1198–1208.
- MACPHERSON, R. & MILLER, J. D. A. (1963). Nutritional studies on *Desulfovibrio desulfuricans* using chemically defined media. *Journal of General Microbiology* **31**, 365–373.
- MILLER, J. D. A., NEUMANN, P. M., ELDFORD, L. & WAKERLEY, D. S. (1970). Malate dismutation by *Desulfovibrio*. *Archiv für Mikrobiologie* **71**, 214–219.
- MILLER, J. D. A. & WAKERLEY, D. S. (1966). Growth of sulphate-reducing bacteria by fumarate dismutation. *Journal of General Microbiology* **43**, 101–107.
- NEUMANN, P. M. (1967). *Investigations into the carbon metabolism and growth inhibition of sulphate-reducing bacteria*. M.Sc. thesis, University of Manchester.
- PEARCE, J., LEACH, C. K. & CARR, N. G. (1969). The incomplete tricarboxylic acid cycle in the blue-green alga *Anabaena variabilis*. *Journal of General Microbiology* **55**, 371–378.
- PEETERS, T. L., LIU, M. S. & ALEEM, M. I. H. (1970). The tricarboxylic acid cycle in *Thiobacillus denitrificans* and *Thiobacillus a2*. *Journal of General Microbiology* **64**, 29–35.
- ROSENBERG, J. C. & RUSH, B. F. (1966). An enzymatic-spectrophotometric determination of pyruvate and lactic acid in blood. Methodologic aspects. *Clinical Chemistry* **12**, 299–307.
- SHIO, I., OTSUKA, S. & TAKAHASHI, M. (1961). Significance of α -ketoglutaric dehydrogenase on the glutamic acid formation in *Brevibacterium flavum*. *Journal of Biochemistry* **50**, 164–165.
- SMITH, A. J., LONDON, J. & STANIER, R. Y. (1967). Biochemical basis of obligate autotrophy in the blue-green algae and thiobacilli. *Journal of Bacteriology* **94**, 972–983.
- STERN, J. R. & BAMBERS, G. (1966). Glutamate biosynthesis in anaerobic bacteria. I. The citrate pathways of glutamate synthesis in *Clostridium kluyveri*. *Biochemistry* **5**, 1113–1118.
- TABITA, R. & LUNDGREN, D. G. (1971). Heterotrophic metabolism of the chemolithotroph *Thiobacillus ferrooxidans*. *Journal of Bacteriology* **108**, 334–342.
- VON TIGERSTROM, M. & CAMPBELL, J. J. R. (1966). Accumulation of α -ketoglutarate by suspensions of *Pseudomonas aeruginosa*. *Canadian Journal of Microbiology* **12**, 1005–1013.
- TRUDINGER, P. A. & KELLY, D. P. (1968). Reduced nicotinamide adenine dinucleotide oxidation by *Thiobacillus neapolitanus* and *Thiobacillus* strain c. *Journal of Bacteriology* **95**, 1962–1963.