ACID FORMATION IN CULTURES OF MYCOBACTERIUM BUTYRICUM

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Smith (1904–1905a, b) was the first to show that human and bovine types of tubercle bacilli gave different reaction curves in glycerol broth. Although both types produced an initial alkalinity, the human type alone gave terminal acidity. Smith (1910) claimed that reaction curves were useful in distinguishing human from bovine types, but atypical curves led subsequent workers to abandon the test. The cause of terminal acidity is problematical. Smith (1910), Long et al. (1922), and Henley and Le Duc (1939) suggested that acids are formed from glycerol. Merrill (1930, 1931a,b), who cultivated pathogenic and saprophytic mycobacteria on broth and on synthetic media, believed that glycerol like available carbohydrates was oxidized completely without the accumulation of intermediate products. These divergent opinions may arise from the use of nitrogenous sources with different effects on glycerol metabolism, or from the use of media with different glycerol/nitrogen ratios (Goyl, 1936; Henley and Le Duc, 1939); and the uncertainty is aggravated by failure to identify the organic acids supposed to accumulate (Dingle and Weinzirl, 1932; Wedum, 1936).

During the routine cultivation of acid fast bacteria on glycerol broth, it was noticed that most species gave a terminal alkalinity, whereas Mycobacterium butyricum regularly showed a terminal acidity. In view of the important bearing acidic products may have on the theory of terminal oxidation in aerobic bacteria, the phenomenon has been investigated in some detail.

MATERIALS AND METHODS

Organisms. The following species from the National Collection of Type Cultures, Lister Institute, London, were examined: Mycobacterium phlei (no. 525), Mycobacterium smegmatis (no. 523), Mycobacterium stercoris (no. 3820), and Mycobacterium butyricum (no. 337).

Cultures. Stock cultures were maintained on 5 per cent glycerol broth for several generations before experimental use. In this paper the term “broth” is used to denote beef infusion to which 1 per cent of peptone and 0.5 per cent of sodium chloride have been added. When another carbon source also has been added, the concentration is given as w/v except in the case of glycerol which is v/v. A synthetic medium (Edson and Hunter, 1943) was used in some experiments. Unless stated otherwise experiments were performed in 100 ml conical flasks containing 20 ml of medium. Cotton stoppered flasks were inoculated by cautious addition of 0.1 mg wet weight of bacteria to the surface (see Edson and Hunter, 1943) and incubated at 38 C in an unsealed condition. Growth (given throughout as mg dry weight) was determined by washing the cell mass three times with distilled water at the centrifuge, transferring to tared crucibles, and drying to constant weight at 100 C. The pH of the medium (initially 7.4) was determined by an indicator method (Lovibond comparator) and checked occasionally by glass electrode measurements.

Determination of acids. α-Ketoglutaric acid was measured by the method of Friedemann and Haugen (1943) on portions of broth adjusted to pH 2.2 (Briggs, 1937) and deproteinized by the addition of 0.1 vol of metaphosphoric acid (10 per cent w/v). Lactic acid and volatile acids were determined by the methods of Friedemann and Graeser (1933) and Friedemann (1938), respectively.

Isolation of 2,4-dinitrophenylhydrazones. 2,4-Dinitrophenylhydrazones of ketonic acids were precipitated from broth cultures (deproteinized as above) and recrystallized either from ethyl acetate or from 40 per cent w/w ethanol.

Manometric measurements. Oxygen uptake was determined by the standard method of Warburg at 38 C. Acid substrates were added as neutral sodium salts. \( Q_o_2 = \mu L O_2 \) consumed per mg dry weight of bacteria per hr.

Bacterial extracts. Extracts were prepared by disrupting the organisms in the Mickle (Hamp- ton, Middlesex, England) disintegrator for 30 min in the cold room at 2 to 3 C. Each tube
contained 4 g of washed bacterial cells, 4 g of no. 12 ballotini glass spheres, and 4 ml of 0.1 m phosphate buffer, pH 7.4. The suspension of disintegrated organisms and glass beads was diluted with 4 ml of buffer per tube, clarified by centrifugation at 1,700 G, and the supernatant dialyzed 24 to 48 hr against distilled water at 2 to 3 C.

Co-carbozylace was a generous gift from Merck and Company, Inc., New Jersey.

RESULTS

Changes of pH accompanying growth. The growth of M. smegmatis, M. stercoris, and M. phlei on broth is poor (maximum about 30 mg in 4 days); M. butyricum shows a long lag period and little growth (7 mg in 21 days); in all cases the medium becomes alkaline. Readily available nitrogen added as ammonium sulfate does not alter the growth or pH curves.

M. smegmatis (figure 1), M. stercoris, and M. phlei grow luxuriantly on 5 per cent glycerol broth and on 5 per cent glucose broth, the pH rising to about 8.4. M. butyricum (figure 1) presents a striking contrast: the maximum growth on glycerol broth is one-third of that attained by the other species, and the pH of the medium falls progressively until growth stops at pH 5.2. On 5 per cent glucose broth M. butyricum is distinguished by a longer lag period, slower growth, and little over-all change in reaction (figure 1).

Although lactate is utilizable, growth on 2 per cent lactate broth is inhibited by the rising pH of the medium, and the final crop is little heavier than that obtained on broth. In 1 and 2 per cent acetate broth, growth of M. smegmatis and M. butyricum likewise is stopped after a few days by rapid rise of pH to 9.1.

Since the striking feature of the curves is the decline of pH in glycerol broth on which M. butyricum is cultivated, an organic acid was sought amongst the metabolic end products.

Isolation of organic acids from cultures of M. butyricum. The organism was grown in 1 L flasks containing 200 ml of 5 per cent glycerol broth.

Lactic acid and volatile acids. Analyses showed that neither lactic acid nor volatile acid was responsible for acidity in the cultures. The small amounts of these substances originally present in the broth disappeared slowly during growth of the organism, e.g., lactic acid in glycerol broth was reduced from 11.9 mg per 100 ml to 0.5 mg per 100 ml after 10 days' growth, and steam volatile acids were not formed.

α-Ketoglutaric acid. An acid was isolated as a lemon-yellow 2,4-dinitrophenylhydrazone which melted at 218 to 219 C after several recrystallizations and did not depress the mp of authentic α-ketoglutaric acid 2,4-dinitrophenylhydrazone. Elementary analysis (Dr. A. D. Campbell, Chemistry Department, Otago University) gave: C, 40.37; H, 3.22; N, 16.86 (calculated C, 40.50; H, 3.09; N, 17.18). Paper chromatographic analysis (Cavallini et al., 1949) confirmed the identity of the derivative and showed that the specimen contained no other dinitrophenylhydrazone. α-Ketoglutaric acid 2,4-dinitrophenylhydrazone could not be isolated from cultures of M. smegmatis, M. phlei, or M. stercoris, all of which produce terminal alkalinity on glycerol broth, or from M. butyricum grown on broth or glucose broth.

Pyruvic acid. Small amounts of pyruvic acid 2,4-dinitrophenylhydrazone were separated from the crude 2,4-dinitrophenylhydrazone (precipitated from 10 day old cultures of M. butyricum) by the aid of a kieselguhr partition column (Howard and Martin, 1950), using butanol saturated with ammonia (3 per cent v/v) as mobile phase and 3 per cent ammonia saturated with butanol as stationary phase. Two distinct
bands were formed. The eluted fractions were dried, dissolved in a small volume of 0.01 m phosphate buffer, and the compounds identified by paper chromatography. The weak diffuse band formed on the column consisted entirely of pyruvic acid 2,4-dinitrophenylhydrazone, and the major band was α-ketoglutaric acid 2,4-dinitrophenylhydrazone. The ratio of α-ketoglutaric/pyruvic derivative was approximately 16/1 in a sample examined quantitatively.

Succinic acid. The medium on which *M. butyricum* had grown was acidified to pH 2.0 and extracted with ether in a Kötscher-Steudel apparatus. A white crystalline organic acid extracted by ether was soluble in water and did not give a 2,4-dinitrophenylhydrazine derivative. The crystals, after washing with ether, melted sharply at 185°C and did not depress the mp of authentic succinic acid. The equivalent weight (by titration) was 60. Both the unknown acid and authentic succinic acid samples gave the same positive fluorescein tests (Mann and Saunders, 1942). The yield of succinic acid in glycerol broth was small (approximately 50 mg per L), and none was found in uninoculated broth media or in cultures of *M. smegmatis*.

These facts identify α-ketoglutaric acid as the chief acid formed during growth of *M. butyricum* on glycerol broth. α-Ketoglutaric, pyruvic, and succinic acids could not be detected in cultures of mycobacteria which rendered the medium alkaline.

Amount of α-ketoglutaric acid formed in glycerol broth. Determination of ketonic acid revealed the accumulation of about 0.3 mg of α-ketoglutaric acid per mg dry weight of bacteria after 10 days' incubation. Figure 2 correlates growth, pH changes, and acid production and shows that the acid is formed throughout the active growth period of the organisms. The amount of ketonic acid produced is almost sufficient to account for the lowering of pH in the medium.

Origin of α-ketoglutaric acid. The meager growth of *M. butyricum* in broth has been emphasized. Growth is stimulated by addition of 0.5 to 1 per cent of glycerol but 5 per cent of glycerol is required to give maximum growth and accumulation of acidic products. This suggests that acid is formed from glycerol until an inhibiting concentration is reached. When the buffering capacity of 5 per cent glycerol broth was increased by addition of 1 per cent potassium phosphate and the initial pH adjusted to the optimum (7.4), the growth amounted to 218 mg in 12 days as compared with 162 mg in the control to which phosphate was not added; and the yield of α-ketoglutaric acid increased correspondingly from 52 to 98 mg. The final pH was 5.5 in both media, but the growth period was prolonged by 8 days as a result of phosphate buffering.

If the peptone constituent of the medium were an important source of α-ketoglutaric acid, it is probable that the amount accumulating would be proportional to bacterial growth, but this is not so. Growth (273 mg) and α-ketoglutaric acid formation (82 mg) attained a maximum in a 5 per cent glycerol broth having a glycerol/peptone ratio (w/w) of 1.6. When less peptone was incorporated in the medium (glycerol/peptone ratios 12.6, 6.3, and 3.2), growth was restricted (130 to 160 mg) but acid formation was constant (about 80 mg).

This conclusion is supported by experiments conducted in 5 per cent glycerol infusion from which peptone was excluded, but the medium was initially buffered to pH 7.4 with 1 per cent potassium phosphate. α-Ketoglutaric acid accumulated in much the same quantity (75 mg) although growth (85 mg in 10 days) was inferior...
to that obtained in peptone media. Chromatographic analysis of the 2,4-dinitrophenylhydrazone isolated from glycerol infusion medium showed it, like former specimens, to consist almost entirely of the α-ketoglutaric acid derivative.

These experiments support the hypothesis that α-ketoglutaric acid originates from glycerol but do not exclude the possibility that some may be derived from the glutamic acid of peptone or from residual amino acids in the infusion.

Formation of α-ketoglutaric acid in a synthetic medium. Small inocula of *M. butyricum* do not grow in a simple synthetic medium in which ammonium sulfate is the sole source of nitrogen and glycerol the sole source of carbon (Edson and Hunter, 1943), but it was found that growth could be obtained by the addition of thiamin to this medium. In eleven days the growth on 100 ml of synthetic medium containing one mg thiamin hydrochloride amounted to 132 mg. The pH fell from 7.5 to 6.0, and 88 mg of α-ketoglutaric acid were formed. The crude 2,4-dinitrophenylhydrazine precipitated from the medium was composed mainly of the α-ketoglutaric acid derivative, but as usual it contained a small quantity of the pyruvic derivative. This experiment shows unequivocally that *M. butyricum* can form α-ketoglutaric acid from glycerol.

Oxidation of glycerol and of organic acids by washed suspensions of *M. butyricum*. Since α-ketoglutaric acid accumulates in cultures of *M. butyricum*, but not in cultures of *M. smegmatis*, grown on glycerol broth, there may be some fundamental differences in their ability to oxidize α-ketoglutarate. Examination of the oxygen consumption of washed suspensions shows that a difference exists (table 1). The oxygen consumption of *M. butyricum* in the presence of α-ketoglutarate is not significantly higher than the endogenous respiration, whereas that of *M. smegmatis* is 64 per cent higher. Both organisms readily oxidize glycerol and acetate; both oxidize glucose, fructose, lactate, and pyruvate at rates consistent with their behavior in culture, but neither organism increases oxygen uptake in the presence of citrate (Edson and Hunter, 1943).

Enzymic reactions in bacterial extracts. The failure of a washed suspension to oxidize a particular substrate does not necessarily exclude the substrate from a role in intermediate metabolism because cells may be impermeable to the substrate. Citrate is a case in point, and α-ketoglutarate may behave similarly. Although citrate is not oxidized by cell suspensions of *M. smegmatis*, it is oxidized to α-ketoglutarate by extracts of this organism (Faine et al., 1951). Dialyzed cell-free extracts of *M. smegmatis* and *M. butyricum* oxidize citrate anaerobically in the presence of methylene blue and magnesium ions. In experiments conducted on a larger scale the product of oxidation was isolated (after deproteinization and removal of methylene blue with charcoal) as a 2,4-dinitrophenylhydrazine and identified chromatographically as the pure derivative of α-ketoglutaric acid. In control experiments with citrate omitted, there was no reduction of methylene blue, and no dinitrophenylhydrazone was obtained.

The ability of extracts to oxidize α-ketoglutarate was examined by the Thunberg technique (methylene blue). Dialyzed extracts of *M. smegmatis* and *M. butyricum* both possess weak α-ketoglutaric dehydrogenase activity which is enhanced by addition of coenzyme A, magnesium ions and diphosphopyridine nucleotide, but the activity of different cultures is variable. α-Lipoic acid was not available. Extracts of *M. smegmatis* and *M. butyricum*
differ in character: the former are water clear after centrifugation at 1,700 G, but the latter remain "milky" due to the presence of lipid-like globules which are not removed at 20,000 G in a refrigerated centrifuge. Owing to this difference and to other uncertainties of extraction, the experiments failed to make a satisfactory quantitative comparison between the rather weak  \( \alpha \)-ketoglutaric dehydrogenases present in the extracts.

**DISCUSSION**

The accumulation of  \( \alpha \)-ketoglutaric acid in culture media has seldom been reported. This acid has been isolated from cultures of *Pseudomonas fluorescens* grown on media containing glucose (Lockwood and Stodola, 1946) or succinate (Kogut, 1952). Using washed suspensions of a strain of *P. fluorescens* which oxidized succinate vigorously, Kogut (1952) observed the accumulation of  \( \alpha \)-ketoglutaric acid accompanied by smaller quantities of pyruvic acid. \( \alpha \)-Ketoglutaric acid has been detected also amongst the products of mold metabolism during deamination of glutamic acid by *Aspergillus oryzae* (Uemura, 1939) and in other reactions. Starved mycelial suspensions of *Penicillium chrysogenum* shaken aerobically with lactate in the presence of arsenite form  \( \alpha \)-ketoglutaric acid (Hockenhull et al., 1951); and felts of *Aspergillus niger*, floated on phosphate buffer containing arsenite, form  \( \alpha \)-ketoglutaric, pyruvic, and dimethylpyruvic acids from endogenous materials, the yield of ketonic acids being increased when glucose is added (Walker et al., 1951).

The evidence presented in this paper shows that  \( \alpha \)-ketoglutaric acid is formed from glycerol but not from glucose when *M. butyricum* is grown on a suitable medium. The mycobacteria catabolize glycerol by a process in which phosphorylation is followed by oxidation to triosephosphate and subsequent breakdown to pyruvic acid (Hunter, 1951, 1953). The condensation reaction of the citric acid cycle has been demonstrated in mycobacteria (Blakley, 1951, 1952; Ochoa et al., 1951), and it has been shown that extracts of *M. butyricum* can oxidize citrate to  \( \alpha \)-ketoglutarate. Based on these facts, the following hypothetical scheme attributes the formation of  \( \alpha \)-ketoglutaric acid from glycerol to a series of reactions, including part of the citric acid cycle:

\[
\text{Glycerol } \rightarrow \text{ triosephosphate } \rightarrow \text{ pyruvate } \\
\downarrow \\
\text{acetyl-coenzyme A } + \text{ oxalacetate } \\
\downarrow \\
\text{ \( \alpha \)-ketoglutarate } \leftarrow \text{ citrate}
\]

*M. butyricum* is a typical acid fast bacillus which has shown no pleomorphism over a period of several years; its colonial and cultural characteristics are typically those of saprophytic mycobacteria. For these reasons it seems unlikely that it has been classified wrongly. It differs from other acid fast saprophytes studied in its distinct glycerophilism and in its ability to accumulate  \( \alpha \)-ketoglutaric acid in glycerol containing media.

The anomalous accumulation of  \( \alpha \)-ketoglutaric acid might be explained by assuming (1) that the complete citric acid cycle normally occurs in mycobacteria (see Edson, 1951), and (2) that the cycle is blocked almost completely at the  \( \alpha \)-ketoglutarate stage in *M. butyricum*. The behavior of washed suspensions (table 1) and the accumulation of small amounts of succinate are consistent with this hypothesis. Kogut (1952) has proposed a similar explanation for the behavior of *P. fluorescens* grown on glucose or succinate medium.

The fact that *M. butyricum* does not form  \( \alpha \)-ketoglutaric acid in glucose broth is not inconsistent because the metabolism of glucose in mycobacteria appears to differ from that of glycerol.

The difficulties encountered in assays of  \( \alpha \)-ketoglutaric dehydrogenase activity have precluded temporarily a crucial test of the hypothesis. Another uncertainty is the source of oxalacetate for the condensation reaction. If the cycle is interrupted, it is necessary to postulate the formation of oxalacetate from pyruvate (small quantities of which accumulate in the medium) and carbon dioxide. *M. butyricum*, unlike *P. fluorescens*, exhibits no obvious ability to oxidize succinate and rather poor ability to oxidize malate and fumarate (table 1).

It is possible that the explanation of the anomaly is more complicated and involves failure to utilize  \( \alpha \)-ketoglutaric acid in assimilations. Preliminary investigation of the growth of *M. butyricum* in glycerol infusion fortified by addition of readily available nitrogen (ammonium sulfate) has shown that luxuriant
growth can occur without accumulation of significant amounts of $\alpha$-ketoglutaric acid. Under some circumstances addition of thiamin to culture media may also prevent accumulation of $\alpha$-ketoglutaric acid. These findings suggest that acid accumulation may be related to the nitrogen metabolism of the organism, and possibly to thiamin deficiency.

Although this study of the behavior of *M. butyricum* raises many unsolved problems, it strengthens the view that reactions of the citric acid cycle play an important role in the metabolism of the mycobacteria.

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**SUMMARY**

*Mycobacterium butyricum*, unlike the other mycobacteria studied, produces terminal acidity (about pH 5.5) in glycerol broth and in glycerol infusion medium. 2,4-Dinitrophenylhydrazones of $\alpha$-ketoglutaric and pyruvic acids and traces of succinic acid have been isolated from the media. The amount of organic acid formed (chiefly $\alpha$-ketoglutaric acid) is sufficient to account for the pH change.

Evidence is presented to show that $\alpha$-ketoglutaric acid originates from glycerol. The evidence is unequivocal in a synthetic medium, but it is possible that some of the $\alpha$-ketoglutaric acid formed in glycerol broth may arise from peptone.

A study of washed suspensions and of cell-free extracts leads to the hypothesis that $\alpha$-ketoglutaric acid is formed from glycerol via pyruvic acid and reactions of the citric acid cycle which is blocked at the $\alpha$-ketoglutarate stage.

**REFERENCES**


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