Why a Co-substrate is Required for Anaerobic Growth of *Escherichia coli* on Citrate

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Citrate was fermented by *Escherichia coli* if a second substrate, such as glucose, lactose or lactate, was available to the organism. The function of the second substrate was to provide reducing power for the formation of succinate from oxaloacetate. Citrate lyase, malate dehydrogenase, fumarase and fumarate reductase were present in cell extracts of *E. coli* at high activity. Oxaloacetate decarboxylase could not be detected, and it is assumed that this lack is the reason for the inability of *E. coli* to grow anaerobically with citrate as the only carbon and energy source.

INTRODUCTION

In 1923, Koser found that *Escherichia coli* and *Enterobacter aerogenes* differ in their ability to utilize citrate. Whereas this compound is readily fermented by *En. aerogenes*, *E. coli* will not grow in a simple medium containing minerals and sodium or potassium citrate (Koser, 1923, 1924). Since then, growth on citrate has been an important criterion for distinguishing between the genera *Escherichia* and *Enterobacter*. Several observations indicated, however, that citrate was not an inert substance to *E. coli*. Ruchhoft et al. (1931) reported that *E. coli* multiplied in Koser's citrate medium but the maximum population levels reached were very low. Also, several isolates which, on the basis of other properties, were classified as belonging to the genus *Escherichia* grew on citrate (Koser, 1924; Parr & Simpson, 1940). Later, it was shown that *E. coli* could decompose citrate under anaerobic conditions if the medium also contained peptone and carbohydrates (Vaughn et al., 1950), and citrate lyase, which is responsible for the anaerobic breakdown of citrate, was detected in *E. coli* and isolated from cell extracts (Dagley, 1954; Wheat & Ajl, 1955).

The role of peptone and carbohydrates in anaerobic citrate utilization by *E. coli* has not been elucidated so far. The results reported in this paper show that *E. coli* is unable to form oxaloacetate decarboxylase and that a co-substrate is required by this organism as electron donor for the conversion of citrate to acetate and succinate.

METHODS

Organism and growth conditions. *Escherichia coli* B (DSM 500; ATCC 23227) was grown under anaerobic conditions in a medium containing (g l\(^{-1}\)): KH\(_2\)PO\(_4\), 0.45; K\(_2\)HPO\(_4\), 0.45; NaCl, 0.9; CaCl\(_2\), H\(_2\)O, 0.02; MgCl\(_2\), 6H\(_2\)O, 0.02; MnCl\(_2\), 4H\(_2\)O, 0.01; CoCl\(_2\), 6H\(_2\)O, 0.01; (NH\(_4\))\(_2\)SO\(_4\), 0.9; Na\(_2\)SeO\(_4\), 5H\(_2\)O, 0.000263; and SL-4 trace element solution (Pfennig & Lippert, 1966), 1 ml l\(^{-1}\). The growth substrates (sodium salts of organic acids or sugars) were added to give final concentrations of 20 mM. Peptone or Casamino acids (0.9 g l\(^{-1}\)) were only added to the medium where indicated.

The Hungate technique (Hungate, 1969) was used for media preparation and for growth experiments which were carried out in 16 ml Belco tubes (Belco Glass, Vineland, N.J., U.S.A.) containing 10 ml medium under a N\(_2\) atmosphere. When the effect of molecular hydrogen on growth was studied, 6 ml H\(_2\),...
was injected into each tube, and the tubes were incubated horizontally on a shaker. Traces of oxygen were removed from N₂ and H₂ by passing them through a vertical Pyrex column packed with copper turnings and heated electrically to approximately 350 °C.

Cells for enzyme determinations were grown in 6 l carboys containing 1-5 l medium. Anaerobic conditions were established by gassing with N₂ for 20 min before inoculation. After 20 h growth the cultures were harvested by centrifugation (20 min, 20000 g, 4 °C). The cells (approx. 2 g wet wt) were suspended in 10 ml 50 mM-potassium phosphate buffer, pH 7-2 containing 3 mM-MgCl₂ and used immediately for extract preparation.

Preparation of cell extracts and enzyme assays. The cell suspensions, which were kept in ice until used, were passed quickly through a French press at a pressure of 78 MPa; cell debris was then removed by centrifugation (30 min, 20000 g, 4 °C). The protein content of the cell extract was determined according to Beisenherz et al. (1953) with crystalline bovine serum albumin as a standard. One unit of enzyme is defined as that amount catalysing the conversion of 1 μmol substrate min⁻¹ at 25 °C.

Citrate lyase activity was determined according to Giffhorn & Gottschalk (1975) and malate dehydrogenase according to O’Brien & Stern (1969). The assay for fumarase was carried out as described by Dorn et al. (1978a) and fumarate reductase activity was determined according to a modification of the method of Kröger & Innerhofer (1976). The assay mixture contained, in a final volume of 1 ml: 50 mM-potassium phosphate buffer, pH 7-2, 0-05 mM-sodium fumarate, 0-025 mM-sodium dithionite and 0-1 mM-benzylojogen. The assay was started by adding 10 μl cell suspension (20-5 mg protein ml⁻¹) after the cuvettes had been flushed with N₂ for 1 min. The oxidation of benzylviologen was followed at 546 nm.

For oxaloacetate decarboxylase the assay of Herbert (1955) was used. Several variations were tried: the K⁺ concentration in the assay mixture was varied between 5 and 20 mM; the K⁺ in the assay mixture was completely replaced by Na⁺; assays were run in the presence of 6 mM-EDTA, 1 mM-MgCl₂ or 1 mM-MnCl₂; the cells tested were grown in media containing either potassium or sodium salts.

NAD- and NADP-specific malic enzyme was measured by the method of Hsu & Lardy (1969) as modified by Dorn et al. (1978a).

Isocitrate lyase and malate synthase were determined according to Dixon & Kornberg (1959).

Determination of substrates and products. Glucose was determined with glucose dehydrogenase and NAD in a reaction mixture containing 108 mM-potassium phosphate buffer, pH 7-6, 135 mM-NaCl, 9 units glucose dehydrogenase ml⁻¹ and 0-2 units mutarotase ml⁻¹ and 2-0 mM-NAD.

Citrate was measured according to Bergmeyer (1974). The fermentation products were determined as described: acetate and succinate (Dorn et al., 1978b); formate (Lang & Lang, 1972); oxaloacetate, D(-)-lactate, pyruvate and ethanol according to the methods of Boehringer Mannheim (1972). The fluorimetric determinations of oxaloacetate were carried out by the method of Williamson & Corkey (1969).

RESULTS

Effect of co-substrates on citrate utilization

The substrates which stimulated the utilization of citrate by *Escherichia coli* under anaerobic growth conditions were investigated. After 24 h growth in appropriate media the absorbance and the final concentrations of citrate in the cultures were determined (Table I). Citrate alone did not support growth and the addition of Casamino acids or peptone had little effect. However, when a fermentable substrate was present in addition to citrate, the latter was degraded and, as judged from the final absorbance of the culture, contributed to growth. Compounds which allowed citrate degradation were glucose, lactose, pyruvate, L-lactate, L-malate plus H₂ and fumarate plus H₂. With formate, H₃ or ethanol as the second substrate, no growth occurred and citrate was not degraded. The growth of *E. coli* on the substrate combination L-lactate plus citrate is noteworthy because neither substrate alone supported growth. Anaerobic growth on L-malate or fumarate, which is stimulated by H₃ (Macy et al., 1976), was further stimulated by citrate. The citrate added to the growth medium was almost completely degraded with glucose, lactose or L-lactate as co-substrate. Some citrate was still detectable after growth with pyruvate, L-malate or fumarate.

Since glucose usually causes catabolite repression with respect to other substrates, the decrease in concentration of glucose and citrate was followed during growth. The results (Fig. 1) demonstrate that both compounds were degraded simultaneously. In addition, experiments with different concentrations of both substrates showed that citrate breakdown
Anaerobic growth of *E. coli* on citrate

Table 1. Dependence of anaerobic citrate degradation by *E. coli* on the presence of a co-substrate

Growth experiments were carried out in Bellco tubes as described in Methods. After 24 h growth the absorbance of the cultures and the citrate concentration in the medium were determined.

<table>
<thead>
<tr>
<th>Carbon and energy sources added</th>
<th>Final citrate concn (mM)</th>
<th>$A_{600}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Glucose</td>
<td>1.5</td>
<td>0.003</td>
</tr>
<tr>
<td>+ Glucose + Casamino acids</td>
<td>1.6</td>
<td>0.94</td>
</tr>
<tr>
<td>+ Casamino acids</td>
<td>0.24</td>
<td>1.6</td>
</tr>
<tr>
<td>+ Glucose + Peptone</td>
<td>1.65</td>
<td>0.24</td>
</tr>
<tr>
<td>+ Peptone</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>+ Lactose</td>
<td>1.005</td>
<td>1.005</td>
</tr>
<tr>
<td>+ Pyruvate</td>
<td>0.48</td>
<td>0.75</td>
</tr>
<tr>
<td>+ Pyruvate + H$_2$</td>
<td>0.48</td>
<td>0.75</td>
</tr>
<tr>
<td>+ L-Malate + H$_2$</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>+ L-Malate + H$_2$</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>+ Fumarate + H$_2$</td>
<td>0.62*</td>
<td>0.62*</td>
</tr>
<tr>
<td>+ Fumarate + H$_2$</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* No growth occurred with citrate and formate, H$_2$ or ethanol.

stopped when glucose was exhausted but that, as expected, glucose breakdown continued after all the citrate had been consumed.

The degradation of citrate by non-growing cells of *E. coli* was also dependent on the presence of glucose or other fermentable substrates. The results summarized in Table 2 show that citrate consumption was low in the absence of glucose but was greatly stimulated by its presence. It is also apparent from Table 2 that the mixed acid fermentation of *E. coli* was shifted to an acetate–succinate fermentation when both glucose and citrate were metabolized. That some citrate was degraded by non-growing cell suspensions in the absence of added glucose was probably due to a product of cell lysis acting as a co-substrate. This is supported by the high carbon recovery of this fermentation. The low isocitrate lyase activity present in these cells (see Table 3) might also have contributed to the slow degradation of citrate and formation of succinate.

These results suggested that it was the function of glucose or other co-substrates to provide reducing power for the conversion of citrate to succinate and acetate. Apparently, one of the products of the citrate lyase reaction, oxaloacetate, could only be converted by *E. coli* into succinate and not into other compounds.

### Lack of oxaloacetate decarboxylase in *E. coli*

Extracts prepared from *E. coli* grown with citrate and glucose were examined for the presence of enzymes involved in anaerobic citrate catabolism (Table 3). Citrate lyase and the enzymes necessary for the reduction of oxaloacetate to succinate were present with considerable activities. Oxaloacetate decarboxylase, however, was not detectable although various assay procedures were tried (see Methods). Furthermore, NAD- and NADP-dependent malic enzyme and the key enzymes of the glyoxylate cycle were present in the cells only at low activities. It seems, therefore, that in *E. coli* there is only one pathway for the further catabolism of oxaloacetate and this is to reduce it to succinate. Additional
Simultaneous utilization of glucose and citrate during anaerobic growth of *E. coli* on these substrates. The mineral medium described in Methods containing 20 mM-glucose and 20 mM-citrate was used. The 61 carboy contained 1.5 l medium and was inoculated with 30 ml of a culture which had been grown anaerobically on the same substrate combination. ○, Absorbance at 600 nm (optical path-length, 1 cm); Δ, concentration of citrate; ○, concentration of glucose.

Excretion of oxaloacetate during incubation of non-growing cells of *E. coli* with citrate. A 10 ml cell suspension in 50 mM-potassium phosphate buffer, pH 7.0 was incubated with 20 mM-citrate under anaerobic conditions at 37°C (○). In a duplicate experiment (○), glucose (final concentration 20 mM) was added after 2 h incubation (arrow). At intervals, samples were removed and oxaloacetate was determined in the cell-free supernatants (see Methods).

**Table 2. Fermentation of citrate and/or glucose by non-growing cells of *E. coli***

Cells from three 10 ml cultures of *E. coli* which had been grown in a mineral medium containing citrate and glucose were centrifuged (5000 g, 20 min), washed twice with 50 mM-potassium phosphate buffer, pH 7.0 and suspended in 10 ml of similar buffer. The resulting suspensions (*A*$_{660}$ = 3.2) were incubated with 200 μmol of the substrates indicated for 4 h at 37°C under strictly anaerobic conditions. The amounts of the substrates and products were then determined as described in Methods.

<table>
<thead>
<tr>
<th>Substrate or product</th>
<th>Citrate + Glucose</th>
<th>Substrate(s):</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>120.3</td>
<td>23.7</td>
<td>74.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>67.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>147.7</td>
<td>23.8</td>
<td>36.2</td>
</tr>
<tr>
<td>d-Lactate</td>
<td>0.5</td>
<td>ND</td>
<td>8.6</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acetate</td>
<td>175.4</td>
<td>28.7</td>
<td>46.8</td>
</tr>
<tr>
<td>Formate</td>
<td>4.4</td>
<td>9.0</td>
<td>36.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>8.7</td>
<td>ND</td>
<td>69.6</td>
</tr>
<tr>
<td>C recovery* (%)</td>
<td>85.8</td>
<td>113.6</td>
<td>98.8</td>
</tr>
</tbody>
</table>

ND, Not detectable. * CO$_2$ was not determined.

Support for this conclusion was obtained from an experiment in which non-growing cells were incubated with citrate and the oxaloacetate concentration in the medium was monitored. As shown in Fig. 2, oxaloacetate was excreted and reached a concentration of approximately 70 μM in the medium. The addition of glucose led to the complete disappearance of this compound.
**Table 3. Specific activities of enzymes possibly involved in the anaerobic degradation of citrate by E. coli**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity [units (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate lyase (EC 4.1.3.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>Malate dehydrogenase (EC 1.1.1.37)</td>
<td>1.55</td>
</tr>
<tr>
<td>Fumarase (EC 4.2.1.2)</td>
<td>0.42</td>
</tr>
<tr>
<td>Fumarate reductase</td>
<td>1.05</td>
</tr>
<tr>
<td>Oxaloacetate decarboxylase (EC 4.1.1.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NAD-malic enzyme (EC 1.1.1.38)</td>
<td>0.07</td>
</tr>
<tr>
<td>NADP-malic enzyme (EC 1.1.1.40)</td>
<td>0.06</td>
</tr>
<tr>
<td>Isocitrate lyase (EC 4.1.3.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Malate synthase (EC 4.1.3.2)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Two catabolic enzymes are required by a number of micro-organisms for anaerobic growth on citrate: citrate lyase and oxaloacetate decarboxylase. The first enzyme cleaves citrate into acetate and oxaloacetate which is subsequently decarboxylated by the second enzyme to pyruvate. The latter then serves as the actual source for the production of ATP and reducing power. Stern (1967) demonstrated that in *Enterobacter aerogenes* both citrate lyase and oxaloacetate decarboxylase are specifically induced by citrate. Both enzymes have been shown to be present in *Rhodopseudomonas gelatinosa* (Beuscher et al., 1974; Bernhard, 1975). Citrate-grown cells of *Streptococcus diacetilactis* (Singh & Srere, 1975; Kummel et al., 1975), *Clostridium sphenoides* (Walther et al., 1977) and *Streptococcus faecalis* (Hiremath et al., 1976) contain citrate lyase and, as judged from their fermentation products, also an oxaloacetate decarboxylase.

A modified pathway for the anaerobic breakdown of citrate is used by *Proteus rettgeri* (Kroger, 1976). In this, a portion of the citrate is cleaved by citrate lyase into acetate and oxaloacetate. Some of the citrate is oxidized via the tricarboxylic acid cycle, and the reducing power thus produced is used for the reduction of oxaloacetate to succinate. Oxaloacetate decarboxylase is apparently not formed by this organism. The results of the present study show that a further modified pathway for the anaerobic breakdown of citrate is employed by *Escherichia coli*. Oxaloacetate decarboxylase is not formed, the tricarboxylic acid cycle is apparently repressed (Amaransingham & Davis, 1965), and the organism has to catalyze a second substrate in order to gain reducing power for succinate formation from oxaloacetate.

The fermentation of citrate to acetate and succinate is only sensible for an organism if the reduction of fumarate to succinate can be coupled to ATP formation by electron transport phosphorylation. Both *P. rettgeri* and *E. coli* have been shown to contain membrane-bound fumarate reductase and to form ATP in this reduction step (Hirsch et al., 1963; Miki & Lin, 1975; Haddock & Kendall-Tobias, 1975; Kroger, 1974; Yamamoto & Ishimoto, 1977; Bernhard & Gottschalk, 1978). *Enterobacter aerogenes*, however, does not form fumarate reductase (Kulla, 1976). This organism relies, therefore, on oxaloacetate decarboxylase for the further catabolism of oxaloacetate via pyruvate and acetyl-coenzyme A. The difference between these two fermentations was noticed by Dagley in 1954 when he observed that non-growing cells of *En. aerogenes* produced acetate, pyruvate and CO₂ from citrate whereas *E. coli* produced oxaloacetate and very little pyruvate.

An alternative route for the formation of pyruvate from oxaloacetate would be the conversion to malate followed by the malic enzyme reaction. Malic enzyme has been shown to be present in aerobically grown *E. coli* (Sanwal & Smando, 1969; Murait et al., 1972). The purified enzyme even exhibits oxaloacetate decarboxylase activity (Iwakura et al., 1979;
Yamaguchi et al., 1973). However, the activity of this enzyme in *E. coli* is so low that it cannot be important for citrate catabolism in this organism. Consequently, the 'reductive pathway' as depicted in Fig. 3 apparently represents the only possible catabolic sequence for the degradation of citrate in *E. coli*.

The results presented in this paper indicate also that *E. coli* is able to form a transport system for citrate under anaerobic conditions. However, the nature and the specific requirements of this system are unknown. The inability of *E. coli* to utilize citrate under aerobic conditions seems to be a problem of transport (Lara & Stokes, 1952). In *E. coli* strains which grow aerobically with citrate, the utilization of this substrate is determined by a plasmid (Smith et al., 1978). *Enterobacter aerogenes*, on the other hand, possesses inducible transport systems for citrate under anaerobic as well as under aerobic conditions (Wilkerson & Eagon, 1972; Johnson et al., 1975).

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**REFERENCES**


An aerobic growth of E. coli on citrate


