OCCURRENCE AND FUNCTION OF ISOCITRITASE AND MALATE SYNTHETASE IN BACTERIA

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The hypothesis has been advanced (Wong and Ajl, 1957; Kornberg and Madsen, 1958) that the significance of isocitritase (Smith and Gunnsalus, 1954; Olson, 1954; Wong and Ajl, 1955; Smith and Gunnsalus, 1957) and malate synthetase (Wong and Ajl, 1956, 1957) in nature stems from the fact that these two enzymes contribute to our knowledge of carbohydrate metabolism. The activities of the enzymes explain the baffling mechanism by which bacteria can grow on two-carbon compounds such as acetic acid. Direct experimental proof for this hypothesis has been lacking. Recently, we have conducted experiments which clearly implicate these two enzymes in the growth of Escherichia coli when acetate is the sole carbon source. The adaptive nature of both isocitritase and malate synthetase has also been further substantiated.

MATERIALS AND METHODS

For the distribution studies, Escherichia coli strain E26 was grown in 8-L quantities on media containing 1.5 per cent of the substrates listed in table 1, (NH4)2SO4, 0.4 per cent; KH2PO4, 0.6 per cent; KH2PO4, 0.2 per cent; MgSO4·7H2O, 0.01 per cent; yeast extract (Difco), 0.2 per cent; initial pH of 7.0. Cells were harvested by Sharples centrifugation after incubation for 24 hr at 30 C with vigorous aeration.

To obtain malate synthetase and isocitritase-deficient cells, E. coli was grown under anaerobic conditions on the glucose-citrate medium previously described by Wheat and Ajl (1955).

The growth curves in these studies were obtained by inoculating a series of 2-L flasks, each containing 1 L of the previously described acetate medium (except that the yeast extract content was reduced from 0.2 to 0.02 per cent), with 2 g each of glucose-citrate grown cells. Initially, and after appropriate time intervals, samples were withdrawn from the flasks and the optical density at 625 mµ determined using a Beckman spectrophotometer (model DU). The number of viable cells was also determined at these times using the conventional spread plate technique. The remainder of the culture was centrifuged and the cells analyzed for malate synthetase and isocitritase activity.

Cell-free extracts were prepared by grinding the cell paste with alumina A-303 according to the procedure of McIlwain (1948). Malate synthetase activity was determined spectrophotometrically in a Beckman recording spectrophotometer (model DK-1) according to the method of Stadtman (1957). The reaction mixture contained, in 3.0 ml, 50 µmoles of Tris (tris(hydroxymethyl)aminomethane) buffer, pH 8; 10 µmoles of MgCl2, and 0.1 µmole of acetyl coenzyme A. Six-hundredths ml of diluted crude extract was added and the change in optical density at 232 mµ recorded for several minutes to detect the possible presence of any acetyl coenzyme A decacylase. Potassium glyoxyxlate, 3 µmoles, was then added and the decrease in optical density recorded. The specific activity of the enzyme is expressed as µmoles of acetyl coenzyme A disappearing per min per mg of protein. Isocitritase activity was measured by the increase in optical density at 324 mµ as a result of the formation of glyoxylate phenylhydrazide. This method has also been used recently by Dixon and Kornberg (1959). The reaction mixture contained, in 3.0 ml, phosphate buffer at pH 7, 200 µmoles; MgCl2, 15 µmoles; phenylhydrazine hydrochloride, 10 µmoles; cysteine hydrochloride, 0.26 µmole; and 0.1 ml of the crude extract. The reaction was initiated by the addition of 10 µmoles of sodium L-isocitrate. The specific activity of the enzyme is expressed as µmoles of glyoxyxlate formed per min per mg protein.

Protein concentrations were determined by

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the method of Lowry et al. (1951). Acetyl coenzyme A was prepared by the method of Simon and Shemin (1953).

RESULTS

Distribution of malate synthetase and isocitritase. Results previously published revealed that malate synthetase could be detected only in cells which had been grown aerobically with acetate as the sole carbon source (Wong and Ajl, 1957). This conclusion was reached on the basis of the assay system used for its determination which depends upon the disappearance of acetyl phosphate in the presence of adenosine triphosphate, coenzyme A, transacetylase, and glyoxylate in the hydroxamic acid reaction. Recent experiments, however, utilizing acetyl coenzyme A and glyoxylate as reactants, and more sensitive analytical procedures, revealed that malate synthetase is indeed present, but in varying concentrations, when E. coli is grown aerobically on a variety of substrates. Data in table 1 clearly indicate that both malate synthetase and isocitritase can be detected when E. coli is grown aerobically on acetate, glycolate, succinate, malate, and ribose. In glucose-grown cells, however, isocitritase could not be detected. Of interest is the finding that the specific activity of isocitritase is consistently below that of malate synthetase. Further, glycolate and acetate-grown cells contain more malate synthetase and isocitritase activity than do cells which are grown on the other substrates.

The data thus far presented raise again the question regarding the adaptive nature of these two enzymes. Since, in the metabolism of all compounds listed in table 1, acetate could be an intermediate and under aerobic conditions further metabolized via mechanisms utilizing both malate synthetase and isocitritase thus causing their adaptive formation, conditions of growth for E. coli were sought which would not involve a further oxidation of acetate even if it were formed in the course of such growth. It appeared that cells grown under anaerobic conditions on a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Malate Synthetase</th>
<th>Isocitritase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolate .</td>
<td>0.882</td>
<td>0.039</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.532</td>
<td>0.055</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.483</td>
<td>0.014</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.344</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Malate</td>
<td>0.169</td>
<td>0.007</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.124</td>
<td>0.006</td>
</tr>
</tbody>
</table>

The data were obtained as follows. Cells were grown anaerobically for 24 hr on the glucose-citrate medium. A portion of these cells were transferred into the acetate medium and allowed to grow aerobically for 24 hr. Enzyme assays were then performed on the cells after 24 hr growth.

Figure 1. Correlation between the enzymatic activities of isocitritase and malate synthetase and the growth of Escherichia coli on acetate. For experimental details see text.
glucose-citrate medium (Wheat and Ajl, 1955) would be suitable for this type of experimental approach. The results are shown in table 2. In E. coli harvested from this type of medium no malate synthetase or isocitritase activity could be detected. Under these conditions the glyoxylate cycle does not operate. When such cells, are subsequently exposed to an acetate medium under aerobic conditions, they do exhibit both of these enzymatic activities. This type of an experiment indicates the adaptive nature of these enzymes. Cells grown anaerobically on glucose-citrate medium contain virtually no malate synthetase or isocitritase; when transferred to an acetate medium and grown aerobically, they adapt and produce both of these enzymes.

**Function of malate synthetase and isocitritase in the growth of E. coli on acetate.** It has been postulated that the major function of malate synthetase and isocitritase is to permit cells to grow on C-2 compounds (Wong and Ajl, 1957; Ajl, 1958; Kornberg and Madsen, 1958). Direct experimental proof for this hypothesis is lacking although data on isocitritase do exist (Kornberg *et al.*, 1958). It appeared that if cells devoid of both malate synthetase and isocitritase were exposed to a medium containing acetate as the sole carbon source, and showed no growth until these two enzymes were produced, the hypothesis concerning their function in C-2 metabolism would be further substantiated. This has been found to be the case. In figure 1 are plotted the specific activities of malate synthetase and isocitritase, and the population curve (turbidity) of cells which have been exposed to aerobic growth with acetate as the sole carbon source. Several observations deserve comment. In cells harvested from the glucose-citrate medium no malate synthetase or isocitritase could be detected. Absolutely no growth takes place for at least 9 hr and no increase in viable cells (table 3) takes place for at least 12 hr during which time considerable amounts of the two enzymes are formed. Thus, isocitritase and malate synthetase increase in specific activity from essentially zero to 0.005 and 0.140, respectively, in 9 hr. It would appear from the data in figure 1 that these are the minimal concentrations required for the initiation of growth since isocitritase begins to appear after 3 hr and malate synthetase after 9 hr on an acetate medium whereas cell division starts only after a 12-hr lag. That the 9-hr lag in growth is due to the lack of isocitritase and malate synthetase is substantiated from the data in figure 2. When the cells are grown aerobically on acetate and subjected to similar experimental conditions as described for figure 1, the lag in the viable cell increase is only 2 hr. The additional conclusion which can be drawn from the data in figure 1 is that isocitritase appears well before any malate synthetase can be detected, a phenomenon to be

TABLE 3  
*Increase in viable cells grown aerobically on an acetate medium*  

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.1 \times 10^9$</td>
</tr>
<tr>
<td>3</td>
<td>$1.0 \times 10^9$</td>
</tr>
<tr>
<td>6</td>
<td>$1.0 \times 10^9$</td>
</tr>
<tr>
<td>9</td>
<td>$1.0 \times 10^9$</td>
</tr>
<tr>
<td>12</td>
<td>$1.0 \times 10^9$</td>
</tr>
<tr>
<td>24</td>
<td>$2.2 \times 10^9$</td>
</tr>
<tr>
<td>36</td>
<td>$7.4 \times 10^9$</td>
</tr>
<tr>
<td>48</td>
<td>$7.2 \times 10^9$</td>
</tr>
</tbody>
</table>

The data were obtained as follows. Cells were grown anaerobically for 24 hr on the glucose-citrate medium. A portion of these cells, devoid of both isocitritase and malate synthetase, were then transferred into the acetate medium and allowed to grow aerobically for 48 hr. Viable cells were determined by conventional spread plate techniques.

*Figure 2.* Increase in the viable cell count of *Escherichia coli* with time in an acetate medium.
expected from what is known concerning the operation of the two enzymes. Malate synthetase requires glyoxylate for the synthesis of malate (Wong and Ajl, 1956). Isocitritase, by cleaving isocitrate, supplies this compound (Olson, 1954). Consequently, it would be expected that isocitritase would be formed prior to malate synthetase. It is important to note also that the rapid synthesis of the two enzymes levels off concomitantly with growth and cell division.

DISCUSSION

The question has been raised as to whether both malate synthetase and isocitritase are adaptive enzymes. From the data presented it appears that both are adaptive since both are present in significant amounts only when the bacteria are grown aerobically on acetate or compounds which may give rise to acetate. When cells are grown aerobically on glucose they contain malate synthetase and virtually no isocitritase. There is no available explanation for this finding except that glucose-grown cells, in addition to providing acetate to the medium, may also provide some source of glyoxylate. This has not been ascertained. The important fact is that cells can be obtained which are essentially devoid of these enzymes and when put into the proper environment will synthesize both of them.

The data on the distribution studies of malate synthetase presented may be assumed to represent the true activities of this enzyme. This assumption is valid since the starting substrates are not acetate, adenosine triphosphate, coenzyme A, transacetylase, and glyoxylate but rather acetyl coenzyme A and glyoxylate. If the former were the case, the final values of malate synthetase might reflect the concentrations not only of malate synthetase, but also of acetate activating enzyme.

The major importance of the malate synthetase and isocitritase reactions stems from the fact that they have been implicated in the mechanisms by which bacteria can grow on C-2 compounds. Although a combination of the activities of these two enzymes provided a plausible hypothesis for this assumption, no direct experimental proof verifies this in the literature. The data presented in this communication provide experimental data on the relationship between time of enzyme formation and the growth and multiplication of cells. We have shown that when cells are grown under conditions where they do not synthesize malate synthetase or isocitritase and are then put in a medium on which their growth presumably depends on the presence of these enzymes, no growth or cell division takes place until sufficient time elapses to allow for their synthesis. When cells are grown under anaerobic conditions in glucose-citrate medium, and are shown to contain neither of the enzymes, and are subsequently grown aerobically on an acetate medium where these enzymes are required before growth and cell division is initiated, a lag period of some 9 hr is required. During this period a great deal of metabolic activity takes place, as exhibited by the production of these enzymes by the cells. Conversely, only a slight lag is required when acetate-grown cells containing malate synthetase and isocitritase are transferred to an acetate medium. The hypothesis is substantiated that malate synthetase and isocitritase are produced by the bacterial cell to afford growth on C-2 molecules as in acetic acid.

SUMMARY

Both malate synthetase and isocitritase are formed when Escherichia coli is grown aerobically on a variety of substrates. The specific activity of malate synthetase is consistently higher than that of isocitritase.

The adaptive formation of both malate synthetase and isocitritase has been again demonstrated.

Experimental evidence has been provided for the hypothesis that the function of these enzymes is to permit E. coli to grow and multiply on acetic acid as the sole source of carbon.

REFERENCES


Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951 Protein measure-
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