STUDIES ON THE MECHANISM OF ACQUIRING CITRIC-ACID-UTILIZING CAPACITY IN ESCHERICHIA COLI

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INTRODUCTION

It has been considered to be of Taxonomical importance that *Aerobacter aerogenes* grows readily in a medium containing only citric acid as carbon source while *Escherichia coli* does not. Vaughn et al\(^1\), however, indicated that *Escherichia coli* was able to utilize citric acid if this acid was added in the culture medium with glucose or some other supplementary compounds. Further, according to Lara and Stokes\(^2\), *Escherichia coli* is capable of oxidizing citric acid in the form of dried cells, though not with living cell suspensions. From these experimental results, they discussed that impermeability of the living *Escherichia coli* cells to citric acid may be responsible for their inability to grow in Koser's citric acid medium. Furthermore, they suggested that the requirement for peptone, glucose or other carbohydrate compounds for citric acid utilization by growing *Escherichia coli* can be explained in terms of the effect of these compounds on cell permeability, in the way that the peptone, glucose, and other carbohydrates provide the energy required to move citric acid across the cell membrane.

This suggestion proposed by Lara and Stokes is thought, however, to be not supported by any positive evidence, and our present paper is to deal with the same subject in detail and make its mechanism clearer.

EXPERIMENTAL METHODS

1. Estimation of citric acid: The procedure described by Natelson, Pincus and Lugovoy\(^3\) was followed to estimate citric acid.

2. Estimation of glucose: The titrimetric measurement proposed by Hagedorn and Jensen\(^4,5\) was employed for glucose estimation.

3. Turbidity determination of bacterial cell suspensions: The turbidity of bacterial cell suspensions was determined by using transparing light with Coleman Junior Spectrophotometer.

4. The strain of *Escherichia coli* employed in our experiments: The strain 18027a (*Escherichia coli* 055 B5) of *Escherichia coli* was used in the whole series of our experiments.
EXPERIMENTAL RESULTS

1. Relationship between glucose and citric acid utilization in cultural experiments

It has been known that citric acid is utilized by *Escherichia coli* only if the medium is given additional supply of glucose. In this case, it is thought that it may give some information about the mechanism of this phenomenon, to clarify when citric acid utilization starts and ceases in comparison with glucose utilization or cell multiplication. Fig. 1 shows this relationship, which indicated that citric acid utilization began approximately at the same time as glucose utilization and the turbidity due to cell multiplication also started to appear at that time.

![Fig. 1 Relationship between glucose and citric acid utilization in comparison with pH and turbidity changes in cultural experiments.](image)

2. Resting cell experiments of citric acid utilization in *Escherichia coli* grown on ordinary media

If, as Lara and Stokes suggest, citric acid utilization is due to the mechanism that the permeability of citric acid through cell membranes is furnished by the energy liberated through glucose utilization, resting bacterial cells of *Escherichia coli* grown on ordinary media should be able to utilize citric acid if both citric acid and glucose are supplied together to them in the medium, because they utilize glucose very readily in resting state, i.e., in non-proliferating state. However, our experiments indicated that the bacterial cell suspen-
sions of *Escherichia coli* did not attack citric acid even in the presence of glucose in the resting state. First, we conducted experiments as follows:

Bacterial cell suspension (ordinary media) in phosphate buffer solution (pH 7.4, 1.15 M) .................. 3.0 ml
Glucose 1% ........................................ 1.0 ml
Citric acid $10^{-2}$ M .................................. 1.0 ml

The mixture of the above mentioned was incubated in a waterbath at 37°C and both glucose and citric acid were estimated in a certain time interval. In the experiments like this, glucose was exhausted very rapidly, mostly in 30 minutes every time, but citric acid never utilized in a long incubation period, namely even in four hours.

In this experiment, the medium did not contain any nitrogenous compounds, so in the next experiments ammonium chloride was supplemented at a concentration of 0.1% to the same medium as above. The experimental results were however the same as before (Fig. 2). In this condition, citric acid was never attacked by the resting cell suspensions of *Escherichia coli* though rapid utilization of glucose present in common. From these experimental results, it seems to be very unlikely that glucose utilization supplies the energy for moving citric acid across the cell membrane.

![Fig. 2 Glucose and citric acid utilization of resting cells, from ordinary culture growth](image)

3. *Citric acid utilization in resting state by Escherichia coli grown on glucose-citrate medium*

Whether the bacterial cells of *Escherichia coli* which have grown on the synthetic medium containing both glucose and citric acid as carbon source, or the ordinary nutrient agar containing citric acid and glucose together, are capable of utilizing citric acid in the presence or absence of glucose in a resting
state, was to be elucidated next. *Escherichia coli* was cultivated on ordinary nutrient agar media containing glucose and citric acid at a concentration of 0.2% and bacterial suspensions were prepared from it for the following experiments.

a. Utilization of citric acid in the absence of glucose.

b. Utilization of citric acid in the presence of glucose.

By the way, the same kind of experiments were conducted with the cells of *Escherichia coli* grown ordinary nutrient agar media. The experimental results are shown in Table 1. It has become herewith clear that either in the presence or in the absence of glucose, the cells of *Escherichia coli* grown in the presence of glucose and citric acid together were capable of utilizing citric acid very readily, while cells grown in the absence of them both did not utilize citric acid at all. The fact that the cells which once had become capable of attacking citric acid, were able to attack citric acid even in the absence of glucose, makes the interpretation unlikely, which the permeability of citric acid across cell membrane is to be due to the energy supply from glucose splitting.

### Table 1. Utilization of citric acid by the cells grown on glucose-citrate medium and on glucose-alone medium

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Cell Substrate</th>
<th>Cells grown on glucose-citrate medium</th>
<th>Cells grown on glucose medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose+ citrate</td>
<td>Citrate alone</td>
<td>Glucose+ citrate</td>
</tr>
<tr>
<td>0'</td>
<td>7.3* μM</td>
<td>7.0* μM</td>
<td>8.2* μM</td>
</tr>
<tr>
<td>30'</td>
<td>0 &quot;</td>
<td>5.0 &quot;</td>
<td>7.6 &quot;</td>
</tr>
<tr>
<td>60'</td>
<td>0 &quot;</td>
<td>1.3 &quot;</td>
<td>6.8 &quot;</td>
</tr>
<tr>
<td>180'</td>
<td>0 &quot;</td>
<td>0 &quot;</td>
<td>6.0 &quot;</td>
</tr>
</tbody>
</table>

* Concentration of citric acid in the reaction mixture

4. *Absence of the active substance affecting citric acid utilization in the growth supernatant from glucose-citrate medium*

It has become clear that the cells themselves, which have grown on citrate-glucose medium, have the capacity to utilize citric acid, but it seemed to us also important to decide whether the supernatant obtained from the culture of *Escherichia coli* glucose-citrate medium contains a substance or substances which enable the cells grown on ordinary nutrient agar to utilize citric acid. At Table 2 shows, the supernatant of the culture of *E. coli* in the medium containing glucose and citric acid together had no activity to render the cells of *E. coli* grown on ordinary agar medium utilize citric acid.
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Table 2. Absence of an active substance affecting citric acid utilization in the growth supernatant from glucose-citrate medium

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Cells grown on ordinary nutrient broth + culture supernatant from glucose-citrate medium</th>
<th>Cells grown on ordinary medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Citrate</td>
</tr>
<tr>
<td>0</td>
<td>1.52 mg/dl</td>
<td>6.0 μM</td>
</tr>
<tr>
<td>0.5</td>
<td>0.51 &quot;</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.54 &quot;</td>
<td>0</td>
</tr>
<tr>
<td>3.0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Concentration of glucose in the reaction mixture
** Concentration of citrate in the reaction mixture

5. Acquisition of the capacity to utilize citric acid in anaerobic growth

When Escherichia coli is cultivated in the medium containing glucose and citric acid, it acquires the capacity to utilize citric acid either in anaerobic or aerobic condition. Table 3 shows this. The cells of Escherichia coli grown in anaerobic condition on an ordinary nutrient agar supplemented with glucose and citric acid at a concentration of 0.2%, also showed as remarkable activity to citric acid as the cells harvested from aerobic growth.

Table 3. Comparison of citrate-utilizing activities of cells from aerobic and anaerobic growths

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Cells from aerobic growth</th>
<th>Cells from anaerobic growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.1 μM</td>
<td>7.5 μM</td>
</tr>
<tr>
<td>90</td>
<td>4.4 &quot;</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>3.9 &quot;</td>
<td>0</td>
</tr>
<tr>
<td>180</td>
<td>2.3 &quot;</td>
<td>—</td>
</tr>
<tr>
<td>240</td>
<td>0.9 &quot;</td>
<td>—</td>
</tr>
</tbody>
</table>

* Concentration of citrate in the reaction mixture
6. Cell proliferation and citric acid utilization

From the above described experimental results, it can be said that we obtain cells of *E. coli* which have become capable of utilizing citric acid by themselves, i.e. with no supply of additional energy, if we cultivate *Escherichia coli* in the medium containing citric acid and glucose together. At this point, one of the important points is whether those cells of *E. coli* having acquired the capacity to utilize citrate are able to grow on citric acid as a sole carbon source. An experiment was conducted to elucidate this problem as follows:

*Escherichia coli* was grown on the ordinary agar medium containing citric acid and glucose at a concentration of 0.2%. The growth was harvested and employed in this experiment. The cells of *Escherichia coli* thus obtained were capable of utilizing citric acid very readily in the resting states as shown in Fig. 3a. The same cells were inoculated into a medium containing citric acid and glucose as carbon source on one hand and into a medium containing citric acid alone. The results are shown in Fig. 3b.

Fig. 3 No proliferation in the citrate-alone medium of cells capable of utilizing citrate.
acid alone as carbon source. Counting of viable cells was made every hour after inoculation. The result was shown in Fig. 3b, indicating that no multiplication took place in the medium containing citric acid alone as a carbon source, but ready proliferation was observed in the one containing both glucose and citric acid together.

Citric acid as a sole carbon source is thus unable to support the growth of *Escherichia coli* cells having already acquiring the activity to utilize citric acid though readily utilized. The idea that citric acid however, is utilized to make materials for bacterial cell bodies, is evidenced by the following experiment. *Escherichia coli* grown on ordinary agar medium was harvested and washed three times with physiological saline. It was inoculated into a) the series of media containing glucose of 0.1% to $0.1 \times 2^{-6}$%, in each with citric acid of 0.2% for all, and b) the same series of media of glucose but will no citric acid. As shown in Fig. 4, cell yields were remarkably increased by the addition of citric acid, that means that citric acid was utilized for synthesizing the materials for bacterial cell bodies.

![Fig. 4 Comparison of cell yields between cultivation in glucose-citric acid medium (○) and glucose medium (●)](image)

Summarizing the results of the above experiments, *Escherichia coli* utilized citric acid when growing in the medium containing both glucose and citric acid together. This is due to the fact that the cells of *Escherichia coli* acquires the capacity to utilize this acid when they grow on citric acid and glucose together as carbon sources. The idea suggested by Lara and Stokes that glucose provides the energy required to move citric acid across the cell membrane of *Escherichia*.
coli organisms is denied because 1) ordinary cells of *Escherichia coli* do not utilize citric acid in resting state in the presence of glucose which in oxidized very rapidly and 2) the cells of *E. coli*, which have only acquired the capacity to utilize citric acid, are able to utilize this acid even in the absence of glucose.

Facing these accumulated facts concerning the utilization of citric acid by *Escherichia coli*, one of the key points of the subject was thought to be the elucidation of how *Escherichia coli* acquires the capacity to utilize citric acid when growing in the medium containing both glucose and citric acid together, and along this line, we first established the working hypothesis that runs as follows:

a. *Escherichia coli* is essentially unable to utilize citric acid supplied from outside but it is always splitting of a mutant cell capable of utilizing citric acid though unable to grow on this acid as a sole carbon source.

b. This mutant cell is splitted off at a considerable mutation rate.

c. This mutant cell grows in the medium containing glucose and citric acid together much more rapidly than the ordinary cell so that the former overgrows soon the latter. This is the selection mechanism for the mutant cell against the normal one.

Our experiments were conducted to examine this hypothesis.

7. Comparison of growth rate of *Escherichia coli* in the glucose medium with and without citric acid

The same numbers of *Escherichia coli* organisms from a broth culture were inoculated after thorough washings with physiological saline into the medium containing both glucose and citric acid together on one hand, and into the

![Fig. 5](image-url) Favorable growth of citrate-utilizing cells in glucose-citrate medium

(●) . . . Growth in the medium containing glucose and citric acid together

(○) . . . Growth in the medium containing glucose alone
medium containing glucose alone on the other hand and the growth rates of both the cultures were compared. The result was shown in Fig. 5, which indicates remarkable difference between both of them. The growth of Escherichia coli in the glucose-citrate medium was remarkably rapid than that in the medium of glucose alone, and we conclude from this result that mutant cells capable of attacking citric acid appear soon during the growth in the glucose-citrate medium after inoculation or contained already in the inoculum (because of the large size of the inoculum in the experiment like this), and the mutant cells overgrow very soon normal cells by utilizing citric acid.

8. Back mutation and its selection

From the above mentioned experimental result, it could be said for the time being that, as our hypothesis claims, the appearance of the mutant cell capable of attacking citric acid from the ordinary Escherichia coli cell and the selective overgrowth of that mutant cell hold true. However, if the mutant cell in question is splitted off at a considerably high rate the ordinary Escherichia coli cell and this mutation is gene-controlled, the mutant cell has to persist in the ordinary cultivation, but ordinarily we do not obtain a clone consisting of the mutant cells capable of utilizing citric acid unless employing the glucose-citric acid medium.

Fig. 6 (a) Inoculation into synthetic glucose medium of cells grown in ordinary medium
Sizes of inoculum: ○ $3.6 \times 10^4$ organisms
○ $1.5 \times 10^4$ ”
○ $0.9 \times 10^4$ ”
We obtained *E. coli* cells capable of utilizing citric acid by cultivating them in the medium containing glucose and citric acid and transferred them further three times successively in the same medium. *Escherichia coli* cells thus obtained were considered to contain citric acid utilizing mutant cell at a very high percentage and there could be only a few proportion of back mutant cells not capable of attacking the acid. After it was confirmed citric acid was very readily utilized by this cell suspension, we inoculated a few of these cells into ordinary broth and examined whether the bacterial cells grown in this broth were able to attack citric acid after though washing with saline. It was not proved that citric acid was attacked.

In this case, if we assume that the backmutation from citric acid utilizing mutant cells occurred at a considerably high rate and this backmutant cells grow more favorably in ordinary nutrient media or others containing no citric acid than the original mutant. An experiment was conducted to know whether or not our above consideration held true. The cells grown on ordinary nutrient agar medium were harvested, washed three times with physiological saline and inoculated into synthetic media containing glucose and salts, in various sizes of inoculum, namely $3.6 \times 10^4$, $1.8 \times 10^4$ and $0.9 \times 10^4$ per each culture tube. In
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the same manner, the mutant cells grown on the nutrient agar added with glucose and citric acid, therefore capable of utilizing citric acid, were inoculated into the same above mentioned medium in inoculum size of $3.2 \times 10^4$, $1.6 \times 10^4$ and $0.8 \times 10^4$. Thus, the growth rate in the synthetic medium containing glucose and salts was compared between both of the above cells of *Escherichia coli*.

Fig. 6 shows the result. The growth of citric acid utilizing mutant cells was remarkably retarded in the medium containing glucose but no citric acid, compared with the ordinary citric acid negative cells. Our consideration concerning backmutation and selection has, we believe, thus been evidenced by this experiment.

DISCUSSION

As for the heritable character of citric acid utilization of *Aerobacter aerogenes*, Ravin (6) has already reported his own experimental results, and along this line, the utilization not only of citric acid but also of other tricarboxylic acid cycle intermediates and furthermore of carbohydrate compounds have been investigated by Ravin (7), or Norman (8). At any rate, we consider it could have been confirmed though some discussions are still remaining, that *Aerobacter aerogenes* splits off at a certain mutation rate a mutant cell not capable of utilizing citric acid which transmits the biochemical characteristic concerning citric acid to its offsprings though with a certain backmutation rate to citric-positive from citric-negative mutants. Therefore, our claim that the mechanism of acquiring citric acid utilizing capacity by *Escherichia coli* is explained by appearance of the mutant cells capable of utilizing citric acid from the original citrate-negative cells, seems to us not to be astonishing.

As has been made clear from our data, it is very unreasonable to conclude, as Lara and Stokes did, that the energy liberated from glucose exidation is required for rendering *E. coli* cells permeable to citric acid from outside. According to our opinion based on the experimental data presented here in this paper, the ordinary cells of *Escherichia coli* are essentially unable to utilize citric acid because of the impermeability of their cell membrane for this acid, but they are intrinsically splitting off a mutant cell of citric acid positiveness at a considerably high mutation rate. The backmutation rate from citric acid positive to negative should be also of a considerably high value, if our mutation hypothesis could hold true. In order that the appearance of mutant cells is made manifest, the selection of the mutant from the group of mother cells is in general a necessary process. In the present case, the selection of the citric acid utilizing mutant cells from the ordinary citric acid negative in a medium containing citric acid together with glucose or other carbon sources on one hand, and the selection of the ordinary citric acid negative cells from the citric acid utilizing mutant cells in ordinary culture media with no citric acid on the other hand, are very remarkable so that only transplantation is enough to obtain a considerably pure clone either of them.
In the above discussion we have used the word "impermeability", but we has only intended to mean with it that *Escherichia coli* has an enzymatic apparatus to utilize citric acid within its cell inside (citric acid activity of cell free extract cells it!), but it can not utilize this very acid. And in this sence, the characteristics of "impermeability" or "permeability" to citric acid is a heritable one for *Escherichia coli*. This is our opinion presented here on the experimental data.

**SUMMARY**

The mechanism of acquiring citric acid utilizing capacity is explained from the hereditary standpoint, namely from the theory of the appearance of the mutant cells capable of utilizing citric acid.

**REFERENCES**