Transport of Succinate in Escherichia coli

I. BIOCHEMICAL AND GENETIC STUDIES OF TRANSPORT IN WHOLE CELLS*

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SUMMARY

A mutant of Escherichia coli unable to metabolize succinate has been used to study the nature of succinate transport in whole cells. It is demonstrated that cells accumulate succinate against a concentration gradient. The uptake of succinate is competitively inhibited by fumarate and malate. The Michaelis constants for the dicarboxylic acids are all in the range of 15 to 30 

The transport system is induced by succinate and repressed by glucose. Mutants lacking adenyl cyclase and some lacking enzyme I of phosphotransferase system acquire succinate transport activity when grown in the presence of succinate and cyclic 3',5'-AMP. Mutants of E. coli unable to transport succinate fall into two broad categories which are referred to as dct (dicarboxylate transport) and ct (carboxylate transport). The dct mutants fail to grow on dicarboxylic acids (fumarate, malate, and succinate) but grow normally on the monocarboxylic acid, lactate. The ct mutants do not grow on either the dicarboxylic acids or on lactate. Both classes of mutants grow well on acetate. The dct class of mutants can be genotypically separated into two categories. The dct A mutants map at about 09 min, and the dct B mutant maps at about 17 min (linked to galactose locus) on the genetic map. The ct mutants are also linked to galactose. It is inferred from these experiments that there are at least two components involved in the transport of succinate in E. coli. It is suggested that the ct gene possibly specifies a carboxylate binding protein. This suggestion is based on the evidence that osmotically shocked cells of E. coli lose the capacity for uptake of succinate.

With the demonstrated existence in recent years of transport systems for almost every compound of metabolic interest in bacteria, the pressing need seems to be the elucidation of the chemical structures and molecular mechanisms that are involved in the transport of metabolites through the limiting membrane. Progress in this area would demand study of transport systems which are amenable to a combined biochemical and genetic approach. The success in the elucidation of the mechanism of group translocation of certain sugars in Escherichia coli cells (1-3) testifies to the suitability of such a combined approach. It is with this aim in view that we initiated studies of succinate transport in E. coli.

Compared to other transport systems, very few studies have been made on the mechanism of transport of di- and tricarboxylic acids in bacteria (4-8). Kay and Kornberg (7, 8) described a dicarboxylic acid transport system in E. coli which is responsible for the uptake of succinate. Unfortunately, however, most of their studies were made on cells in which metabolism of succinate after uptake was not prevented by suitable genetic or inhibitor blocks. Kay and Kornberg (7, 8) did show, however, that a component determined by dct (dicarboxylate transport) gene was probably required in the transport process.

Genetic studies of various workers have indicated that the phosphotransferase system of Roseman (2) may also be somehow involved in the transport and utilization of succinate. Thus, E. coli strain MM 6 lacking enzyme I (pts I) of the phosphotransferase system is not only defective in the utilization of glucose, maltose, and some other sugars but also is unable to grow on succinate (9-11). Similar observations have been made in other E. coli strains lacking some components of the phosphotransferase system (12). It is quite possible that the defects in these cases are not directly related to transport and metabolism of succinate but mediated indirectly by cyclic AMP, as has been shown for the metabolism of carbon sources other than glucose in enzyme I defective mutants (13, 14).

In order to be able to undertake and interpret cell-free studies of succinate transport at the molecular level we considered it desirable to clarify first the following questions in whole cell studies. What is the extent of involvement of phosphotransferase system in succinate uptake? How many genetically defined components are involved in transport? What is the nature of these components?
tecture of transport process (kinetics, substrate specificity, etc.) in cells in which transport and catabolism of succinate are separated?

**EXPERIMENTAL PROCEDURE**

*E. coli* K-12 Strains—All of the strains used in the present work with their relevant genetic characteristics are listed in Table I.

Media—The following media were used. Medium A was a minimal salts medium (16) with citrate omitted. It was supplemented with amino acids and other growth factors, wherever required, at a concentration of 25 μg per ml. Unless otherwise specified, carbon source was added at a concentration of 4 mg per ml. LB medium consisted of 1% Bacto tryptone, 0.5% yeast extract, and 1% NaCl. The solution was neutralized to pH 7.0 with NaOH. LB agar was LB medium solidified with 1.1% Bacto agar. Cells were optimally induced for succinate transport by addition of 85 mM succinate to LB medium.

Growth of Cells—All bacterial strains were grown under vigorous agitation on a gyratory shaker at 37°C. Cell growth and cell concentration was determined with a Klett-Summerson colorimeter using Filter 66. Strain CB 11 was used to calibrate the readings of optical density with wet and dry weight determinations.

Genetic Methods—Transductions with coliphage P1 were performed according to Lennox (17). The procedure used for bacterial matings was based on the method of Curtiss et al. (18). The mating pairs were separated by shaking in the isab saw apparatus of Low and Wood (19). Mutants were selected by mutagenization with N-methyl-N′-nitro-N′-nitrosoguanidine (20) followed by enrichment using penicillin (21). Hfr strains were cured of the F factor, when required, by growth in the presence of sodium dodecyl sulfate (22).

**Isolation of Mutants Defective in Succinate Transport**—Three different methods were used to isolate mutants defective in the transport of succinate and the other two dicarboxylic acids, fumarate and malate.

Method I—This method was essentially that used by Kay and Kornberg (8). Approximately 10^8 bacteria were plated without prior mutagenization on minimal medium containing 0.4% acetate (as the sole carbon source) and 0.05 mM 3-fluoromalate. The papillae that arose after 48 hours incubation at 37°C were picked up and single colony isolations were made by restreaking on fluoromalate-containing medium.

Method II—Cells grown on LB medium were mutagenized and expressed for a few generations in Medium A containing acetate (0.4%). The cells were then centrifuged, suspended in Medium A containing 0.4% succinate, and subjected to penicillin selection. Mutants were plated on acetate minimal medium and subsequently replica plated on succinate-containing medium. Colonies that did not grow on succinate were picked and purified.

Method III—This method, referred to as tritium suicide method, was based on the assumption that in a mixed population of cells, those able to transport tritiated succinate would be killed while those unable to do so would survive. Log phase cells were mutagenized and allowed to recover in minimal medium containing acetate. The cells were then exposed to 1.5 mCi of sodium [2,3-3H]succinate per ml for 90 min at 37°C. The cells were then washed twice with cold Medium A, suspended in acetate minimal medium, and stored at 4°C. At various time intervals (24 hours, 48 hours, etc.) samples were taken and spread on LB-succinate (0.4%) medium. After 16 hours, plates with well isolated colonies were replica plated on to LB-succinate (0.05 mM sodium succinate) medium containing 1.5 μCi of sodium [2,3,4-3H]succinate. These were incubated at 37°C for 5 hours. A filter paper was then carefully pressed on top of the colonies. It was removed, dried, and exposed to a No Screen Kodak x-ray film. On the developed film wild type colonies appeared as dark circles while the succinate transport negative colonies appeared greyish. The latter type of colonies were picked from the original plate and purified. The radiographie method has in the past been used by Zwaig and Lin (23) and others (24) to identify transport deficient mutants for other metabolites.

**Enzyme Assays**—For enzyme assays cells were grown overnight in the required medium, washed with 0.1 M Tris-Cl buffer, pH 7.0, and sonicated. After centrifugation, the supernatant or the pellet (suspended in a small amount of Tris-Cl buffer) was used to measure enzyme activities. Succinate dehydrogenase (EC 1.3.4.3) was measured in the supernatant by the method of Wright and Sanwal (28). P-Enolpyruvate carboxykinase (EC 4.1.1.32) and malate dehydrogenase (EC 1.1.1.37), and malic enzyme (EC 1.1.1.40) were measured in the supernatant by the method of Enzymology (27). Fructose diphosphatase (EC 3.2.1.14) was measured in the supernatant by the graphic method has in the past been used by Zwaig and Lin (23). The latter type of colonies were picked from the original plate and purified. The radiographie method has in the past been used by Zwaig and Lin (23) and others (24) to identify transport deficient mutants for other metabolites.

**TABLE I**

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<tr>
<th>Strain designation</th>
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<tr>
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<td>dct B, leu, Sm</td>
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*a Probable genotype (carbohydrate transport).*
Presence of fumarate reductase was tested using the method of Hanber and Singer (30). The specific activities of the various enzymes were calculated on the basis of micromoles of product formed per min.

**Succinate Metabolism in Vivo**—Cells grown in LB medium plus 1% succinate were harvested at log phase. The cells (3.1 mg dry weight) were washed twice and suspended in 5 ml of 0.05 M phosphate buffer pH 7.5. Sodium [2,3,4-14C]succinate (17.4 μCi) was added to the cells to a final concentration of 0.05 mM and the suspension was incubated at room temperature for 10 min. After incubation the cells were harvested by filtration through Millipore filters. The filter was immediately transferred to boiling water. After 15 min the filter and debris were removed by centrifugation and the supernatant was evaporated to dryness in an Evapo-mixer. The residue was redissolved in 200 μl of distilled water. Thirty microliters of the extract was applied to MN300G cellulose (polygram cel 300) thin layer plate and subjected to chromatography using a solvent consisting of ethanol, ammonia, and water in the proportion of 90:20:10. Autoradiography was performed by exposure of the plates to Kodak x-ray film (No Screen) for 7 days.

**Succinate Transport Assay in Whole Cells**—Cells grown in LB medium plus 1% succinate were harvested at log phase. The cells were washed twice and suspended in 0.05 M phosphate buffer pH 7.5. Cell concentration was adjusted to 0.64 mg dry weight per ml. In typical experiments, 5 ml of 0.10 M sodium [2,3,4-14C]succinate were added to 5 ml of the cell suspension. Reagents and cells were held at 23° before and after mixing. At various time intervals, aliquots of 1.5 ml were filtered through Millipore filter and washed with 5 ml of 0.05 M phosphate buffer pH 7.5. The whole procedure was completed within 10 s. The Millipore filter was then dissolved in 5 ml of Bray’s scintillation fluid (31). The radioactivity of the sample was counted in a scintillation counter. The amount of [2,3,4-14C]succinate adhering to the bacterial cells was measured by using cells pretreated with formaldehyde (32). This amount was subtracted from the experimental values.

**Cell Shocking Procedure**—Cells were osmotically shocked by the method of Heppel (33). Log phase cells were washed with 0.05 M phosphate buffer pH 7.5. The cell pellet was suspended in 80 parts of 20% sucrose containing 0.03 M Tris-HCl pH 7.2 and 0.1 M EDTA. The cells were stirred gently for 10 min at room temperature and centrifuged. The cells were then rapidly dispersed by vigorous stirring in 80 parts of cold distilled water. The shocked cells were centrifuged again and resuspended in 0.05 M phosphate buffer.

**Chemicals**—Except where otherwise indicated, all chemicals were obtained from commercial sources and were of the highest available purity. [2,3,4-14C]Succinic acid (23 mCi per mmole) and [2,3,4-14C]succinic acid (100 mCi per mmole) were obtained from Amersham/Searle Co. Dithionium fluoromolate was a generous gift from Dr. Paul Kent (Oxford). Pentylmalonate and n-butylmalonate were gifts from Dr. R. Williams (Toronto). 2-Phenylsuccinic acid was donated by Dr. S. I. Chavin (Durham). Showdomycin was a gift from Shionogi Research Laboratory (Japan). Membrane filters (0.45 μm) were obtained from Schleicher and Schuell Inc.

**RESULTS**

**Isolation of a Strain for Succinate Uptake Studies**—Essential for studies of succinate transport is the availability of a strain of *E. coli* which is unable to catabolize succinate to any significant extent. The major enzymes immediately involved in the catabolism of succinate are the two succinate dehydrogenases, one of which also functions as a fumarate reductase (34). A mutant lacking succinate dehydrogenase and fumarate reductase was made by a two step procedure. Hfr H was mutagenized and a mutant lacking succinate dehydrogenase (sdh) was selected by penicillin enrichment. The sdh mutants do not grow on minimal succinate medium but grow readily on fumarate minimal medium. From a sdh mutant a strain was selected which had lost the capacity to grow on fumarate as a sole carbon source and which was also unable to grow on glycerol plus fumarate anaerobically (a test suggesting lack of fumarate reductase, frd, Reference 34). The sdh, frd markers (which are linked) were transferred to the female strain W 604 (gal) by mating and a gal+ sdh, frd recombinant was selected. This strain (CB 11) had normal levels of all of the enzymes required for succinate metabolism (for a list of enzymes assayed, see “Experimental Procedure”) except succinate dehydrogenase and fumarate reductase. The specific activities of the enzymes assayed in glycerol grown, stationary phase cells of a wild type organism (W 604) were 0.06 for succinate dehydrogenase and 0.008 for fumarate reductase. In strain CB 11, under identical growth conditions the specific activities were 0.0012 and 0.001, respectively.

**Absence of Succinate Catabolism in CB 11**—For studies of succinate transport it was important to show that succinate taken up by CB 11 cells remains essentially unchanged. Cells in which the succinate transport system had been induced (see later) were allowed to take up [14C]succinate for varying lengths of time and extracted in boiling water. Thin layer chromatography of extracts revealed that over 95% of the radioactivity in different experiments was present in a spot whose RF value was the same as the reference [14C]succinate (Fig. 1). Some very faint spots revealed by autoradiography (Fig. 1) were probably due to slight contamination of the commercial [14C]succinate utilized in our experiments. In contrast to our results, Kay and Kornberg (7) have demonstrated that wild type as well as the sdh mutants of *E. coli* metabolize succinate rapidly to glutamate.

**Inducibility of Succinate Transport**—Earlier studies of other workers (7,8) have demonstrated that succinate is taken up much faster by *E. coli* cells which have been grown on succinate as the sole carbon source; this induction of transport, however, is sensitive to catabolite repression by glucose. Since it is well known that succinate will induce a number of enzyme systems responsible for the metabolism of succinate (26, 35, 36) and since in previous studies (7) no attempt was made to use cells in which catabolism of succinate was prevented, we decided to re-examine this question using strain CB 11. The results of this experiment are summarized in Fig. 2. It may be noted that the highest accumulation of succinate is achieved in CB 11 cells grown on succinate and least in cells grown on glucose. In comparison to CB 11, the apparent amount of succinate taken up in induced cells of Hfr H (wild type) or CB 10 (sdh) is extremely high (Fig. 3). This is understandable in view of the fact that in wild type or *sdh* strains there is a metabolic drag on the succinate taken up through the transport system.

Since the succinate transport system is repressed by growth of cells on glucose (Fig. 2), the question arises whether this is due to catabolite repression (37). Pastan and Perlman (13, 38) have shown that most glucose sensitive enzymes require cyclic AMP and a cyclic AMP receptor protein for their induction. It is also known (39, 40) that glucose lowers the concentration of cyclic AMP in the cells by unknown mechanisms, thus repressing the

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2 Lo, Rayman, and Sanwal, unpublished observations.
The synthesis of inducible systems. The relationship of cyclic AMP to the induction of succinate transport was investigated in strains MP 259 (lacking adenylate cyclase) and CB 13 (Table I). The latter strain was constructed from CB 11 by introducing it via conjugation the cya gene of the Hfr strain MP 259.

Results presented in Fig. 4 show that the succinate transport system is induced in CB 13 only when the cells are grown in succinate and cyclic AMP. Each of these compounds added alone into it via conjugation the cya gene of the Hfr strain MP 259.

Results presented in Fig. 4 show that the succinate transport system is induced in CB 13 only when the cells are grown in succinate and cyclic AMP. Each of these compounds added alone into it via conjugation the cya gene of the Hfr strain MP 259.

Results presented in Fig. 4 show that the succinate transport system is induced in CB 13 only when the cells are grown in succinate and cyclic AMP. Each of these compounds added alone into it via conjugation the cya gene of the Hfr strain MP 259.

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Results presented in Fig. 4 show that the succinate transport system is induced in CB 13 only when the cells are grown in succinate and cyclic AMP. Each of these compounds added alone into it via conjugation the cya gene of the Hfr strain MP 259.
to the growth medium has no inducing effect. Again, as is evident for the wild type strain in Fig. 3, MP 259 shows apparent succinate uptake rates much higher than CB 13. This is very probably due to the simultaneous induction with the transport system of enzymes which metabolize succinate and create a drag on the accumulated succinate. Cyclic AMP added at a concentration of 5 to 10 mM to uninduced cells of CB 13 during uptake experiments had no effect on succinate transport.

Absence of Involvement of Phosphotransferase System—As was mentioned earlier, many of the pts I mutants, lacking enzyme 1 of the phosphotransferase system reported in the literature, show a simultaneous lack of ability to grow on glucose, some other sugars, and succinate. The extent of involvement of pts in succinate uptake was investigated in strains MM 6 and 903 C. In strain MM 6, it was first established that all of the enzymes involved in succinate metabolism (succinate dehydrogenase, fumarase, malate dehydrogenase, P-enolpyruvate carboxykinase, malic enzyme, succinic thiokinase, and fructose diphosphatase) were present but the activity of the phosphotransferase system, measured by the tolenuzation procedure of Gachelin (41), was almost nonexistent. Accumulation of succinate in MM 6 was also negligible in transport assays. If the absence of succinate transport system in MM 6 was due to pts I mutation it was expected that revertants of the pts locus would regain in one step the ability to grow on glucose as well as succinate. Spontaneously occurring mutants of MM 6 were, therefore, selected on glucose minimal and succinate minimal media. The characteristics of these revertants are shown in Table II. Four points are worthy of note in this table. First, MM 6 is unable to grow on the three dicarbonylic acids, malate, fumarate, and succinate. Second, revertants selected separately for growth on glucose (MM 6-G) and succinate (MM 6-S) are unable to grow on the dicarbonylic acids and glucose, respectively. Third, MM 6-G grows well with acetate or lactate as the sole carbon source which suggests that the functioning of tricarbonylic cycle and oxidative phosphorylation is normal in these strains. Fourth, the over-all activity of the phosphotransferase system, as measured by α-methyl glucoside phosphorylation by permeabilized cells (41), is very much depressed in MM 6 and MM 6-S, but is present at near normal levels in the revertant (MM 6-G) which can grow on glucose but not on the dicarboxylic acids. These results support the conclusion that pts is not involved in succinate transport and that MM 6 harbors another mutation which specifies a component of succinate (and, possibly other dicarboxylic acids, see Reference 7) uptake system. This locus designated det B (soon later) can be transferred from MM 6 by transduction to other E. coli strains (Table IV). In one experiment P. plage was grown on MM 6 (gal+, det B) and the plage lysate was used to transduce strain W 604 (gal K, det B+). Gal+ recombinants were selected. Out of 4.108 gal− transductants 21 were found to have inherited the det B locus. Thus, the latter is linked to gal K. As expected from the location of pts on the genetic map (42), none of the gal+ recombinants inherited pts I. That the strains carrying det B lack the capacity to transport succinate and other dicarboxylic acids is documented later.

The second strain 903 C carrying pts P and unable to grow on succinate behaves differently than MM 6. Fastan and Perlman (13) have shown that addition of cyclic AMP to some pts I mutants restores their ability to be induced for a number of inducible systems. Since succinate uptake is mediated by an inducible system it seemed possible that cyclic AMP may restore succinate uptake and metabolism in strain 903 C. This was indeed found to be the case. Cells grown in 10 mM cyclic AMP and succinate show considerable uptake of succinate, while cells grown without cyclic AMP are unable to transport it to any significant extent.

Characteristics of Succinate Transport—Fig. 2 shows the time dependence of succinate transport. The amount of succinate accumulated is linear for at least 3 min and a steady state is reached at about 20 min. Assuming that the volume of cell water is 2.7 μl per mg dry weight (43), it can be readily calculated that after 10 min the concentration of succinate inside the cell is 7.71 mM when the outside concentration is 0.05 mM. Thus, succinate is taken up against a concentration gradient which implies an active process.

Another argument for the uptake of succinate as being active comes from the study of temperature dependence of uptake and exit of succinate. Many active transport systems have high temperature coefficients, since energy metabolism is itself highly temperature dependent. As Fig. 5 shows at 0° only 0.4 nmoles of succinate is taken up by cells; at 15° the cells start taking up succinate actively and reach a maximum at around 36°. There is a 44-fold increase in uptake from 0° to 36°. This large increase of uptake and the nonlinear pattern of increase preclude the fact that succinate transport is a passive process, which has a low temperature coefficient. The decrease in succinate accumulated in the cells (Fig. 5) at higher temperatures may be both due to an inactivation of the uptake system and an increase in the rate of exit. The figure also illustrates the fact that succinate exit from preloaded cells is temperature dependent.

Michaelis Constant for Succinate and Competitive Inhibition by Dicarboxylic Acids—To determine the $K_m$ of succinate, initial rates of uptake were measured at different concentrations of succinate. L-Malate and fumarate were found to be competitive inhibitors of succinate uptake. This is illustrated in Fig. 6. We have done competition experiments with wide concentration ranges of fumarate and malate to determine the inhibition constants but have not presented the results here. The $K_m$ for succinate is 10 to 20 μM and the $K_i$ for both malate and fumarate are in the range of 20 to 30 μM. The competition experiments support the contention of Kay and Kornberg (7) that the dicarboxylic acids are not substrates for uptake but are competitive inhibitors of succinate uptake.
boxylic acids are all taken up by the same transport system in
E. coli.

Inhibition of Succinate Transport—Results obtained with the
use of energy poisons show (Table III) that the respiratory chain
and possibly oxidative phosphorylation are required for succi-
inate transport. Inhibition by azide, cyanide, arsenate, and
dinitrophenol particularly show that metabolic energy is required
for the uptake process. All the sulfhydryl reagents tested show
some inhibitory effects on succinate uptake. This may indicate
that sulfhydryl groups are involved in succinate transport.

It has been postulated by Chappell (44) that succinate enter-
ing mitochondria does so in exchange for malate. This process
is carried on through the functioning of a dicarboxylate trans-
porter. This transporter is specifically inhibited by some su-
cinate analogues, such as 2-phenylsuccinate (45), n-butyl-
amalonate and pentylmalonate. An attempt was made to see if
there are any similarities in this regard between the mitochondrial
and bacterial dicarboxylate transport systems. As seen in Table
III, the mitochondrial inhibitors are without any significant ef-
fect on the succinate uptake system of E. coli.

Effect of Osmotic Shocking of Cells on Succinate Transport—It
is well known that EDTA-treated E. coli cells when osmotically
shocked show impairment of some transport systems (see review
in Reference 33). This impairment has generally been considered
to be due to a physical damage to the cell membrane caused by
the shocking procedure.

We have attempted to reconstitute the transport system by
adding back concentrated shock fluid to the shocked cells.
These efforts have consistently failed. Recently, we have suc-
ceeded in isolating a succinate-binding protein from the shock
fluid. The purification and properties of this binding protein
will be the subject of a later communication.

Genetic Elements Involved in Succinate Transport—Before mean-
ingful attempts at chemical isolation and eventual reconstitution
of succinate transport components are made we considered it
worthwhile to get an indication of the number of components
involved in this transport. This is best done by isolation of
mutants defective in succinate transport. This approach has al-
ready been used successfully, for example, in the study of sulfate
(46) and histidine (47) transport. In order to obtain mutants
in as many succinate transport components as possible, we have
used three different methods of selection, which are discussed in
detail under “Experimental Procedure.” The characteristics of
these mutants (all independent isolates) including one (604 G)
recovered by transduction from strain MM 6 (see Table I) are
listed in Table IV. The mutants can be grouped into two classes
phenotypically. All the fluoromalate-resistant mutants (CB 19,
CB 20 and CB 21) and strain 604 G are unable to grow on the
dicarboxylic acids but grow on lactate (dct mutants). The rest
of the mutants, obtained by tritium suicide and penicillin selec-
tion method, do not grow on lactate or the dicarboxylic acids (ct
mutants). Both of these two phenotypic classes of mutants
grow well on acetate which shows that they do not lack any en-
zymes involved in the metabolism of succinate (or other dicar-
boxylic acids) and components of the respiratory chain. Each of

![Fig. 7. Effect of osmotic shock on uptake of succinate by strain CB 11. Log phase cells grown in LB plus 1% succinate were washed and resuspended in 0.05 M phosphate buffer pH 7.5 (0.64 mg dry weight per ml). The suspension was divided into two equal aliquots. One aliquot was osmotically shocked as described in the text. The shocked cells were resuspended in the original volume of buffer. Uptake experiments were then performed with both shocked and unshocked cells by standard methods.](http://www.jbc.org/)

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<td>Iodoacetate</td>
<td>10^{-4} M</td>
<td>79</td>
</tr>
<tr>
<td>Showdomycin</td>
<td>10^{-4} M</td>
<td>87</td>
</tr>
<tr>
<td>2,2'-Dithiodipyridine</td>
<td>10^{-3} M</td>
<td>65</td>
</tr>
</tbody>
</table>
| **Competitive inhibitors of mito-
  chondrial dicarboxylate trans-
  porter**                       |               |            |
| 2-Phenyl succinate               | 5 x 10^{-4} M | 0          |
| N-Butylmalonate                  | 5 x 10^{-4} M | 10         |
| Pentylmalonate                   | 5 x 10^{-4} M | 1          |

TABLE III

Effect of various inhibitors on succinate uptake by strain CB 11

Cells were preincubated with inhibitor for 15 min at 23°, and
uptake experiments were then performed as described under
"Experimental Procedure." Cells were allowed to take up sodium
[2,3-14C2] succinate for 10 min before filtration. All the inhibitors
were made up in aqueous solutions.
TABLE IV
Some growth characteristics of the mutants of E. coli defective in dicarboxylic acid transport with their probable locus assignments on the chromosome

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutant isolation method</th>
<th>Growth on succinate, fumarate, or malate</th>
<th>Growth on lactate</th>
<th>Growth on acetate</th>
<th>Genotype assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB 15</td>
<td>Tritium suicide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ct+</td>
</tr>
<tr>
<td>CB 16</td>
<td>Fluoromolate resistance</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>CB 17</td>
<td>Fluoromolate resistance</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ct±</td>
</tr>
<tr>
<td>CB 18</td>
<td>Fluoromolate resistance</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>Hfr H</td>
<td>Fluoromolate resistance</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>dct A+</td>
</tr>
<tr>
<td>CB 19</td>
<td>Fluoromolate resistance</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>CB 20</td>
<td>Fluoromolate resistance</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ct±</td>
</tr>
<tr>
<td>CB 21</td>
<td>Fluoromolate resistance</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>UC 11</td>
<td>Penicillin selection</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ct+</td>
</tr>
<tr>
<td>CB 22</td>
<td>Fluoromolate selection</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>CB 23</td>
<td>Fluoromolate selection</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>CB 24</td>
<td>Fluoromolate selection</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ct±</td>
</tr>
<tr>
<td>W 604</td>
<td>Transduction from MM 6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>dct B+</td>
</tr>
<tr>
<td>604 G</td>
<td>Transduction from MM 6</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>dct B</td>
</tr>
</tbody>
</table>

* Probable genotype.

The mutants listed in Table IV is a point mutation, as judged by the fact that each can be back mutated and such revertants acquire the ability similarly to grow on all three dicarboxylic acids and lactate. In addition, each can be transduced by phage F, grown on wild type strain Hfr H and the transductants again are able to grow well on sucinate, fumarate, malate, and lactate. When the mutants are grown on LB plus succinate medium (to induce the succinate transport system) and subsequently tested for their capacity for the transport of succinate, it is found that none of them are capable of accumulating succinate to any significant extent. Results of some representative experiments are illustrated in Fig. 8.

Assuming that one gene determines one separate component (regulatory or structural) of the transport system, we have tried to get an idea of the number of components involved by determining whether the mutated locus in each of the succinate transport-deficient strains is the same or different. The approximate position of the mutated locus in fluoromolate resistant mutants was determined by first curing these strains of the F factor by sodium dodecyl sulfate method (22) and using these as females in interrupted matings with the multiauxotrophic Hfr strain UC 118 which donates its chromosome counterclockwise starting at about 62 to 64 min. Recombinants were selected on minimal media containing succinate as the sole carbon source (which also counterselected the Hfr due to the absence of added growth factors). In all fluoromolate resistant mutants the succinate marker was transferred early (approximately 10 min). It is thus very likely that the genetic defect in these strains corresponds to the dct gene (herein referred to as dct A) described by Kay and Kornberg (8).

Fig. 8. Succinate uptake by various dicarboxylate transport negative mutants. The following strains were used: 1, CB 18; 2, CB 22; 3, 604 G; 4, CB 20. Parental strains Hfr H and CB 18, and strain CB 11 are also shown as controls. Table IV gives details of the phenotypes of the various strains employed in this experiment.

The gene defects (ct) in strains obtained by penicillin enrichment (CB 22, CB 23, and CB 24) were mapped by transduction procedure described earlier for strain MM 6. In a typical transduction experiment using CB 22 (gal+, ct) as the donor and W 602 (gal, ct+) as the recipient, 886 gal+ transductants were picked up and the frequency of occurrence of succinate-negative phenotype in these was scored. About 52% linkage with gal K was obtained. In a reciprocal transduction experiment where UC 118 (gal, ct+) was used as a donor and CB 22 as the recipient, ct+ was used as a selected marker and gal as an unselected marker. Of the 420 ct+ colonies picked up, 191 colonies were gal K. So the linkage between ct of strain CB 22 and gal K is 45%. Strains CB 23 and CB 24 were not mapped as extensively as CB 22, but transduction results show a linkage of 40 to 50% with gal K. Cursory examination of mutants obtained by the tritium suicide technique suggests that they may be similar to CB 22 but this has to be confirmed by detailed mapping experiments.

The dct locus of strain MM 6, as was indicated earlier, seems to be also linked to gal K, but phenotypically it is entirely different from CB 22 (Table IV). We show in the accompanying communication (48) that MM 6-G, as well as the fluoromolate resistant mutants, lack a membrane component required for the transport of succinate. The locus defective in MM 6 is designated here as dct B.

DISCUSSION

It has become clear from recent studies of tricarboxylic (4-6) and dicarboxylic acid transport in various bacteria, including E. coli (7, 8), that the transport systems are generally inducible and sensitive to catabolite repression by glucose (6, 37). In E. coli, thanks to the work of Perlman and Pastan (13, 38), it is now firmly established that inducible enzymes responsible for the catabolism of diverse sugars and other substrates require cyclic AMP as a part of positive control system for their induction. In the present work we have been able to establish that cyclic AMP is also involved in the induction of succinate transport system. This requirement for cyclic AMP possibly also explains the puzzling finding in the literature (9-12) that mutants lacking en-
zyme I of the phosphotransferase system (2) are unable to grow on succinate as the sole carbon source. For unknown reasons, some enzyme I-defective mutants show enhanced catabolite repression which can be antagonized by cyclic AMP (13). In addition, we have shown that lack of succinate uptake (and consequent lack of growth on this carbon source) in strain MM 6, one of the earliest pleiotropic psl I mutants described in the literature (9), is due to a second unlinked mutation involved in succinate transport (det B). It is, thus, quite clear that the phosphotransferase system is not primarily involved in any way in the succinate uptake process.

Most transport systems described so far in bacteria (1), including the tricarboxylic acid transport in Bacillus (6), are involved in the concentrative uptake (active transport) of metabolites, i.e. transport occurs against a concentration gradient. Some exceptions are, however, known. Glycerol, for instance, seems to be transported in E. coli by facilitated diffusion and captured by ATP-dependent phosphorylation (49, 50). Group translocation systems for some sugars are also known (3).

Nevertheless, it is surprising that Kay and Kornberg (7) in their study of dicarboxylic acid transport in E. coli found very little concentration uptake of succinate in whole cells. Their studies were, however, performed with cells in which metabolism of succinate was not prevented by suitable genetic blocks. The studies presented here clearly show that the steady state concentration ratio of intracellular to extracellular succinate can reach as high as 154 (Fig. 2). In confirmation of these results obtained with whole cells, we show in the accompanying paper (48) that membrane vesicles, devoid of enzyme systems metabolizing succinate, also accumulate succinate against a concentration gradient. The inhibition of succinate uptake in whole cells by energy poisons and uncouplers of oxidative phosphorylation (Table III) point to the conclusion that transport of succinate is an active process.

The kinetic parameters evaluated for succinate transport are $K_m = 14 \mu M$ and $V_{max} = 20 \mu moles \times min^{-1} \times g$ dry weight$^{-1}$. The latter value is smaller than is expected for most substrates which can be utilized as single carbon sources by bacteria (51). Kay and Kornberg (7) have found an approximately similar value for $V_{max}$ for all dicarboxylic acids transported by their E. coli strains.

It seems probable from our results and those obtained by others (7, 8) that the succinate transport system is also utilized by cells for the uptake of fumarate and malate. The evidence rests primarily on the demonstration that fumarate and malate cause competitive inhibition of succinate and that mutants unable to accumulate succinate fail to transport or utilize for growth the other two dicarboxylic acids. From competition experiments such as those presented in Fig. 6, the $K_m$ for fumarate and malate can be calculated to be about 20 to 30 $\mu M$, a value somewhat higher than that found for succinate.

Genetic evidence presented here suggests that in addition to a component which has been described by Kay and Kornberg (8) earlier, at least one other component is involved in dicarboxylic acid transport. The gene loci governing the synthesis of these components are referred to as det A and det B, the former mapping at about 60 min (8) and the latter at around 17 min (near gal) on the E. coli map (42). Evidence obtained with mutants made by trisulide suicide and other techniques indicates that a third component maps near gal, and distinct from det B, may also be involved in the uptake process.

The nature of these mutants, referred to here simply as ct, is puzzling because in addition to the lack of transport activity for succinate these strains fail to grow on lactate, a monocarboxylic acid. It is entirely possible that lactate and the dicarboxylic acids share one common transport component (regulatory or structural), but this point remains to be investigated. Transport systems described in the literature vary with regard to the number of components that are involved in vivo. Thus, phosphate (52) and lactose (53) uptake systems seem to be essentially one component systems while sulfate (46) and histidine (47) transport are mediated by several components working in unison. It is still unclear how the different elements act in facilitating transport, but it is evident that progress in this area will depend upon a complete understanding in biochemical terms of the individual components. Results presented in the accompanying communication (48) suggest that det A and det B loci are responsible for the synthesis of membrane-bound elements involved in a dicarboxylic acid uptake. The role of ct gene is not clear, but it may be that this locus is involved in specifying a carbohydrate-binding protein. This statement, at the moment, is entirely speculative but is based on our finding that whole cells subjected to osmotic shock lose the capacity for succinate uptake. In addition, we have partially purified from shock fluids a non-enzyme protein which binds succinate tightly.

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