CITRATE TRANSPORT SYSTEM OF *STREPTOCOCCUS DIACETILACTIS*

R. J. HARVEY AND E. B. COLLINS

Department of Food Science and Technology, University of California, Davis, California

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ABSTRACT

HARVEY, R. J. (University of California, Davis) AND E. B. COLLINS. Citrate transport system of *Streptococcus diacetilactis*. J. Bacteriol. **83**:1005-1009. 1962.—The uptake of citrate by *Streptococcus diacetilactis* is mediated by a transport system that was distinguished from passive diffusion by inducibility and kinetics of uptake. In these characteristics the system is similar to the β-galactoside permease of *Escherichia coli*. The citrate transport system of *S. diacetilactis* differs from β-galactoside permease by requiring metabolic energy for operation under conditions that do not permit intracellular accumulation of the substance transported.

Acetoin formation by species of *Streptococcus* and *Leuconostoc* proceeds only when the pH of the medium is below 6.0 (Long and Hammer, 1936; Beynum and Pette, 1939). That citrate metabolism is necessary for acetoin production by these organisms suggests that citrate uptake could be similarly dependent on pH. This was shown to be the case in the present study. Though this effect of pH is consistent with uptake of citrate by passive diffusion, data show that the uptake of citrate by *S. diacetilactis* (Matuszewski et al., 1936) is mediated by a transport system somewhat similar to that described for β-galactosides in *Escherichia coli* (Rickenberg et al., 1956).

MATERIALS AND METHODS

The cultures used, *S. diacetilactis* DRCI (Swartling, 1951; Collins and Harvey, 1962) and *L. citrovorum* CAF1, were propagated routinely in sterile skim milk fortified with 0.75% nonfat milk solids. For preparation of cell suspensions, cultures were grown for 16 hr at 26 C in lactose broth with 2% sodium citrate · $3H_2O$ added. To ensure maximal activity of the transport system, an additional 1% sodium citrate was added after the 16-hr growth period, and cultures were incubated an additional hour. Cells were harvested by centrifugation, washed twice in cold tris(hydroxymethyl)aminomethane chloride buffer (0.05 M; pH 7.3), suspended in the same buffer at a dry weight of about 30 mg/ml, and held at 0 C. When cell suspensions were treated with toluene, the method was that of Gerhardt et al. (1953)

Since citritaseless mutants of *S. diacetilactis* and *L. citrovorum* were unavailable, accumulation of citrate in cells could not be expected. Thus, we measured citrate uptake at 30 C by determining the rate of disappearance of citrate from the suspension medium. Samples (about 2 ml) were removed from a reaction mixture at intervals, and cells were removed from the samples by filtering with a type AA Millipore filter. The filtration was complete in 5 to 10 sec. Duplicate samples of the filtrate were analyzed for citrate by the method of Marier and Boulet (1958).

Chloramphenicol was obtained from Parke, Davis and Co., Detroit, Mich. Commercial crystalline reagent-grade sodium citrate, sodium arsenate, and 2,4-dinitrophenol were used.

RESULTS

Citrate utilization by intact and toluene-treated cells. Rate of citrate disappearance will represent entry of citrate into cells rather than breakdown by intracellular citritase only if entry is rate-limiting. That was demonstrated to be the case by comparing the rates of citrate utilization by intact and toluene-treated cells of *S. diacetilactis*. At pH 5.0, rates for toluene-treated cells were 1.5 to 3.0 times those for intact cells. In intact cells the difference between rate of entry and breakdown by citritase may be even greater, since the pH optimum for citritase is 7.5 (Harvey and Collins, 1961).

Effect of pH on citrate uptake. Figure 1 shows the rates of citrate uptake by intact cells of *S. diacetilactis* and *L. citrovorum* over the pH range of 4.5 to 7.0. Rates increased rapidly below pH 6.0, showing that the fully ionized form of citrate did not enter the cell readily. This is consistent with citrate uptake by passive diffusion, but it

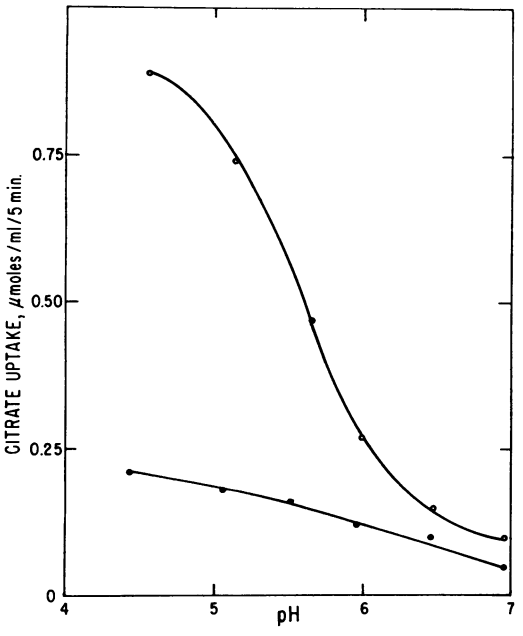

FIG. 1. *Effect of pH on uptake of citrate by intact cells of Streptococcus diacetilactis and Leuconostoc citrovorum. Reaction mixtures contained: sodium citrate, 2 × 10⁻³ M; MgSO₄, 5 × 10⁻³ M; phosphate buffer, ionic strength 0.2; cells; total volume, 20 ml. ○ = S. diacetilactis, 1 mg cells/ml; ● = L. citrovorum, 2 mg cells/ml.*

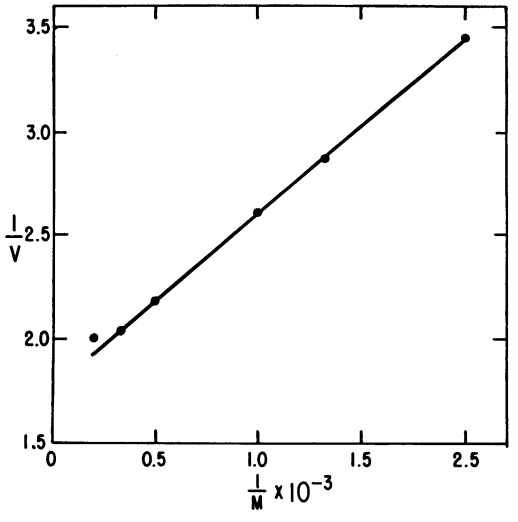

FIG. 2. *Effect of citrate concentration on citrate uptake by intact cells of Streptococcus diacetilactis. Reaction mixtures contained: sodium citrate, 0.25 × 10⁻³ to 5.0 × 10⁻³ M; MgSO₄, 5 × 10⁻³ M; phosphate buffer, 0.37 M, pH 5.0; cells, 0.63 mg dry wt per ml; total volume, 20 ml. Units of V are μmoles per ml per 5 min per mg dry wt cells.*

does not exclude the operation of a pH-dependent permease-type system. Further studies to distinguish the two possibilities were carried out using only *S. diacetilactis*, with the assumption that citrate probably enters *S. diacetilactis* and *L. citrovorum* cells by the same mechanism.

Effect of citrate concentration on citrate uptake. Rate of citrate uptake by passive diffusion should be directly proportional to citrate concentration. But the response of a permease system to substrate concentration should follow Michaelis-Menten kinetics and yield a linear double-reciprocal plot. In view of this difference, the effect of citrate concentration (over the range of 0.25-5.0 × 10^{-3} M) on rate of citrate uptake by intact cells was determined. The double-reciprocal plot of the data (Fig. 2) is clearly linear, and can be taken to indicate that a catalytic system is involved in the transport of citrate.

Uptake of citrate by cells grown in the absence of citrate. Further distinction from passive diffusion could be made if citrate transport were found to be inducible. Thus, *S. diacetilactis* was grown in citrate-free broth and harvested as previously described. Uptake of citrate by the cell suspension was compared to citrate breakdown by the same cells after toluene treatment, and to uptake by citrate-grown cells. Figure 3 shows that the kinetics of uptake by citrate-grown cells were essentially zero-order, with a slight lag before the maximal rate was reached. The rate of uptake by cells grown in the absence of citrate was low initially and increased with time. Results with the toluene-treated cells showed citritase to be constitutive (Harvey and Collins, 1961) and nonlimiting in the uptake of citrate, indicating that the adaptation to citrate uptake must represent induction of a transport system rather than induction of citritase. The presence of 50 μg/ml of chloramphenicol prevented the uptake of citrate by cells grown in the absence of citrate, did not affect uptake by citrate-grown cells, and did not affect the activity of citritase. The results thus show that citrate is transported by an inducible system, and that protein synthesis is necessary for establishing the system.

Effect of lactose and 2,4-dinitrophenol on citrate transport. The rate of citrate uptake by fully induced intact cells was increased 2.3 times by concurrent metabolism of 2 × 10^{-4} M lactose

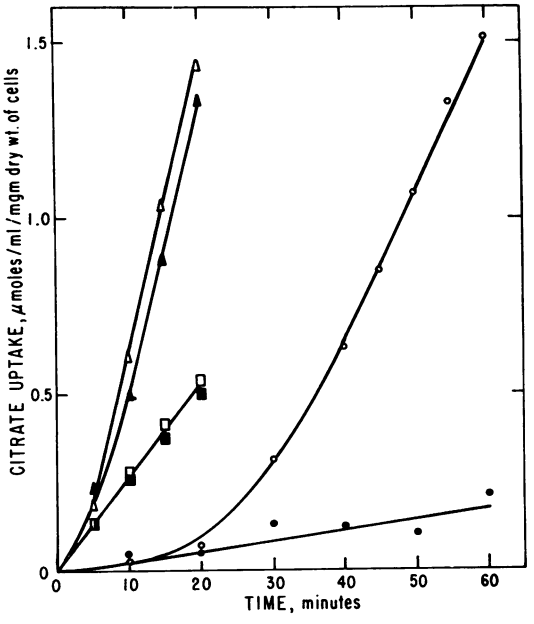

FIG. 3. *Inducibility of citrate transport in Streptococcus diacetilactis. Reaction mixtures contained: sodium citrate, 2 × 10⁻³ M; phosphate buffer, 0.44 M, pH 5.0; MgSO₄, 5 × 10⁻³ M; cells, 0.65 mg dry wt/ml; total volume, 20 ml. Cells were incubated for 30 min in the reaction mixture before addition of citrate at zero time.* △ = *citrate grown cells;* ○ = *cells grown in the absence of citrate;* □ = *cells grown in the absence of citrate, toluene treated. Open symbols = no chloramphenicol; closed symbols = chloramphenicol, 50 μg/ml.*

(Fig. 4). This effect was reversed by 2,4-dinitrophenol. With no lactose present, 2,4-dinitrophenol reduced the rate of citrate uptake to zero. Citritase activity was unaffected by the 2,4-dinitrophenol, as indicated by the toluene-treated controls. In these experiments the reaction mixtures were buffered sufficiently to prevent the treatments from producing a change in pH.

Qualitatively, sodium arsenate produced the same effects as 2,4-dinitrophenol, but effective concentrations of arsenate interfered with the estimation of citrate. The results, being somewhat erratic, are not presented.

Influence of 2,4-dinitrophenol on yield of cells from lactose. In lactic streptococci, conversion of lactose to lactic acid via the glycolytic pathway provides the major source of adenosine triphosphate (ATP). In glycolysis, arsenate reduces the net synthesis of ATP by competing with phosphate in the triose dehydrogenase reaction. But the uncoupling action of 2,4-dinitrophenol is usually considered to be associated with oxidative phosphorylation. Interpretation of the data in Fig. 4 in terms of energy requirements of the citrate transport system requires that 2,4-dinitrophenol interfere with the energy metabolism of lactic streptococci.

Cell yields for a number of different organisms (grown anaerobically on a variety of energy sources) have been found proportional to the theoretical amounts of ATP synthesized from the energy source (Bauchop and Elsden, 1960). The average value was 10.5 g dry wt of cells per

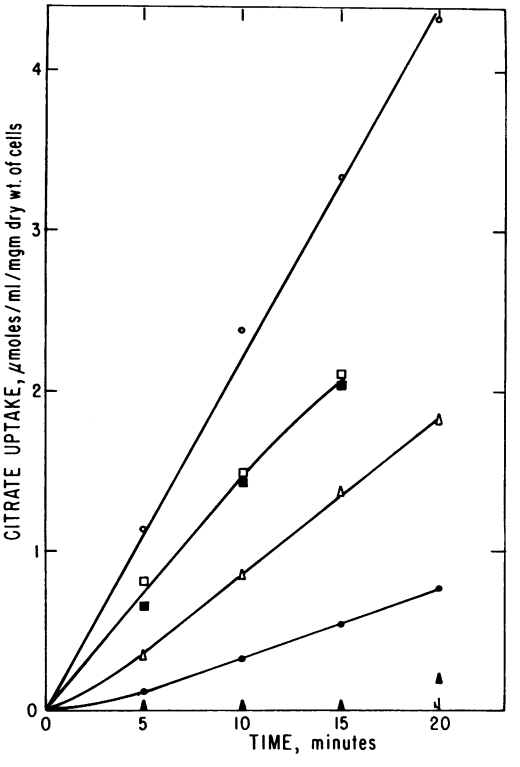

FIG. 4. *Uptake of citrate by cells of Streptococcus diacetilactis in the presence of lactose and 2,4-dinitrophenol. Reaction mixtures contained: sodium citrate, 10⁻³ M; MgSO₄, 5 × 10⁻³ M; phosphate buffer, 0.075 M, pH 5.0; cells, 0.21 or 0.53 mg dry wt/ml; total volume, 20 ml. Cells were incubated in the reaction mixture for 30 min before addition of citrate at zero time. Lactose was added at zero time.* △ = *intact cells;* □ = *toluenized cells;* ○ = *intact cells, plus 2 × 10⁻⁴ M lactose. Open symbols = no 2,4-dinitrophenol; closed symbols = 2,4-dinitrophenol, 10⁻³ M.*

mole of ATP, with a range of 8.3 to 12.6. According to this relationship, the effect of uncoupling of phosphorylation should be reduction of the yield coefficient. Figure 5 presents results showing that 2,4-dinitrophenol does reduce the yield coefficient of *S. diacetilactis*.

The yield coefficient for *S. diacetilactis* was 49 g of cells/mole of lactose. This corresponds to 12.3 g of cells/mole of ATP, assuming complete conversion of the lactose to lactic acid via the glycolytic pathway. In the presence of 10^{-3} M 2,4-dinitrophenol, the yield coefficient was reduced to 9.5 g of cells/mole of lactose, indicating an availability of only 0.8 mole of ATP per mole of lactose metabolized.

Some unknown constituent of the basal medium provided an energy source, since limited growth occurred in the absence of lactose. The magnitude of growth in the "blank" was not greatly affected by 2,4-dinitrophenol, indicating that this compound probably does not produce general inhibition of growth.

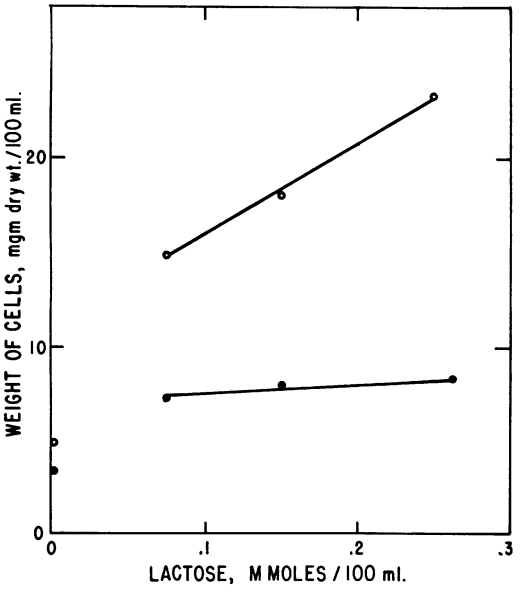

FIG. 5. *Influence of 2,4-dinitrophenol on the cell yield of Streptococcus diacetilactis with lactose limiting. Basal medium: peptone (1%), yeast extract (1.5%), KH_2PO_4 (0.05%), $MgSO_4$ (0.02%), and sodium acetate (0.21%). Lactose and 2,4-dinitrophenol were added as filter-sterilized solutions. Cultures were incubated in screw-capped bottles at 26 C for 70 hr and harvested by centrifugation. Cells were washed twice in distilled water, washed into tared dishes, dried under vacuum at 70 C, and weighed.* ○ = *control, no 2,4-dinitrophenol;* ● = 10^{-3} M *2,4-dinitrophenol.*

That lactose was metabolized in the presence of 2,4-dinitrophenol was determined by two methods: by analyzing the culture medium for lactose after the cells had been grown and harvested, and by measuring directly (by electrometric titration of the lactic acid produced) the rate at which resting-cell suspensions glycolyzed lactose. The latter method revealed that 10^{-3} M 2,4-dinitrophenol slightly increased the rate of glycolysis.

DISCUSSION

Citrate transport systems have been indicated for certain *Pseudomonas* species, by a lag period before oxidation of citrate by intact cells, and by prolongation of the lag period by amino acid analogues, ultraviolet irradiation, and chloramphenicol (Kogut and Podoski, 1953; Barret, Lakon, and Kallio, 1953; Clarke and Meadow, 1959). A similar situation has been shown for *Aerobacter aerogenes* by Green (Davis, 1956), who found that glucose prevented adaptation to citrate. Data of the present study show that the entry of citrate into *S. diacetilactis* cells is mediated by a similar inducible system. In kinetics of uptake and inducibility, the citrate transport system of *S. diacetilactis* is identical with the β-galactoside permease of *E. coli* (Rickenberg et al., 1956).

The yield coefficient of *S. diacetilactis* with limiting lactose was decreased by 2,4-dinitrophenol, though this compound did not prevent the uptake and glycolysis of lactose. Therefore, 2,4-dinitrophenol must act (in some unspecified manner) either to reduce the net synthesis of ATP or to make unavailable the ATP produced. The data can thus be interpreted in terms of the energy requirement of the permease system, assuming that the system was not affected directly by lactose, arsenate, or 2,4-dinitrophenol.

Though it is difficult to reach definite conclusions about the intracellular concentration of citrate during the experiments, it is probable, since citritase activity was nonlimiting, that transport was not taking place against a concentration gradient. If energy were required only for accumulation, as appears to be the case with the β-galactoside permease of *E. coli* (Cohen and Monod, 1957), the rate of citrate uptake should not have been affected by lactose, 2,4-dinitro-

phenol, or arsenate. Since 2,4-dinitrophenol and arsenate reduced the rate to zero, and since this effect was partially reversed by lactose, it must be concluded that the citrate transport system of *S. diacetilactis* requires a source of metabolic energy for operation even with the extracellular concentration of citrate exceeding the intracellular concentration. This same interpretation appears reasonable for the results of Leach and Snell (1960), who found that 2,4-dinitrophenol prevented the transport of amino acids and peptides into cells of *Lactobacillus casei*. In the absence of an exogenous energy source, energy must be provided from endogenous reserves. Conclusions cannot yet be drawn as to the mechanism of energy coupling in this system.

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