

Induction and Properties of the Citrate Transport System in *Aerobacter aerogenes*

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The mediated transport of citrate in *Aerobacter aerogenes* was studied. According to data obtained by examining the distribution of radioactive citrate at room temperature and at 0 C, a carrier system appears to be located on the membrane. The carrier system is inducible and very specific, not acting on the related compounds isocitrate and cis-aconitate. Induction required synthesis of both ribonucleic acid and protein as determined by starving auxotrophic mutants and by using specific inhibitors of protein synthesis. Citrate transport was inhibited by *N*-ethyl maleimide, dinitrofluorobenzene, and uranyl nitrate. A kinetic study of uranyl nitrate inhibition revealed that the inhibition of citrate transport was different from that of glucose penetration. Cyanide also discriminated citrate from glucose penetration inhibiting only the former. These last results suggested that energy is required for citrate penetration.

Davis (9) reported that *Aerobacter aerogenes* metabolized citrate after a lag period. The lag was due to the induction of a permeability system, the enzymes necessary for citrate metabolism being constitutive. A similar situation has been described in *Pseudomonas* (4, 5, 19) and in *Streptococcus diacetilactis* (14).

These systems, where only the synthesis of the membrane carrier is required to metabolize a substrate, may have advantages as model systems compared to systems in which the induction process involves the synthesis of both the membrane carrier and the metabolic enzymes. The most thoroughly studied example of this last type is the β -galactoside permease- β -galactosidase operon of *Escherichia coli* (15).

For this reason we considered it interesting to extend the studies of Green and Davis and to obtain further information on the citrate-transport system of *A. aerogenes*. The present study was undertaken to investigate the chemical nature and general properties of this membrane carrier.

MATERIALS AND METHODS

The following strains were used in this study: *A. aerogenes* strain ENCB from our collection; *A. aerogenes* strain 1143 Ou⁻, a uracil auxotroph obtained from F. M. Harold, National Jewish Hospital, Denver, Colo.; and *A. aerogenes* Ou⁻ Arg⁻ (22) obtained from strains 1143 by nitrosoguanidine treatment.

All the strains were maintained on slants of a basal

medium containing 0.7% K₂HPO₄, 0.3% KH₂PO₄, 0.01% MgSO₄·7H₂O, 0.2% (NH₄)₂HPO₄, and 1.5% agar. Glucose was sterilized separately and added to 0.5% concentration. Uracil and arginine were added to a concentration of 100 μg/ml each for the growth of the mutants. When necessary, citrate was substituted for glucose in the medium. Flasks (1 liter) containing 200 ml of medium were inoculated from the slants and shaken for 12 hr at 28 C. Cells were harvested by centrifugation, washed three times with 0.05 M potassium phosphate buffer (pH 7.3), and resuspended in the same buffer.

Growth was measured turbidimetrically in a Klett photocolormeter with a red (660 nm) filter. Dry weight was calculated from the turbidity by using an appropriate calibration curve. Citrate penetration was measured by oxygen uptake in the presence of citrate as substrate. The oxygen uptake was measured by conventional manometric techniques (27) at 37 C.

In some experiments citrate uptake was measured by use of citrate-1, 5-¹⁴C. Cells were incubated with 2 μmoles of citrate (0.05 μc/mmole) in a final volume of 5 ml of phosphate buffer. At intervals, 1-ml samples were removed, rapidly chilled, and filtered through membrane filters (0.47 μm pore diameter; Millipore Corp., Bedford, Mass.). The filtered material was recovered and the cells were washed with ice-cold water. The whole procedure took less than 2 min. Radioactivity of the cells and the dried filtrate was measured with a gas-flow counter (Nuclear Chicago model 186A). The radioactivity was corrected for self-absorption by extrapolation to zero thickness and was expressed as counts per minute per milliliter of cell water or of supernatant liquid. The intercellular vol-

ume was measured with inulin and gave a value of 18.8% under our standard conditions. The volume of intracellular water was 83.8% of the cell volume.

To identify the radioactive material accumulated, cells were incubated with citrate-1,5- ^{14}C for 5 min; the cells were filtered, washed with cold 0.1 M KCN, and resuspended in 5 to 10 ml of water. The suspension was heated in a boiling-water bath for 5 min and filtered again. The cells were dried and counted. The filtrate was freeze-dried and resuspended in 150 μl of water, from which samples were taken to measure radioactivity. The remainder of the material was subjected to paper electrophoresis by using a pyridine-formate buffer, pH 3.5 (28), at 6 v/cm for 6 hr. The paper was dried and cut in 2-cm pieces, and radioactivity was counted. Citrate-1,5- ^{14}C was simultaneously run as a marker on a different sheet of paper.

Cell-free extracts were obtained by breaking the cells with a 9-kc Raytheon sonic oscillator for 10-min periods. The extracts were centrifuged at 15,000 $\times g$ for 15 min in an HR-1 International centrifuge, and the residue was discarded.

Iscitrate dehydrogenase was measured by the method described by Kornberg (20), and citratase was measured by the method of Daron and Gunsalus (8). For the determination of aconitase, the method of Anfinsen (3) was modified as follows. In a quartz cell of 1-cm path length, 0.02 ml of 0.03 M potassium cis-aconitate, 0.1 ml of extract, and enough 0.05 M phosphate buffer (pH 7.3) to bring the volume to 3.0 ml were mixed, and the change of absorbancy at 240 nm was measured. Dehydrogenase activity was measured by following the reduction of 2,6-dichlorophenol indophenol (DCPIP) in the presence of various substrates, as described by Ells (10) for succinic dehydrogenase. In all cases, the change in absorbancy was measured in a Beckman DU spectrophotometer coupled to a Photo-volt Varicord model 43 recorder. Enzyme activity was expressed as change in absorbancy per minute per milligram of protein.

Protein was measured in the extracts by the method of Warburg and Christian as described by Layne (21).

RESULTS

The three strains of *A. aerogenes* studied oxidized citrate only after a lag phase. Induced cells, on the other hand, utilized citrate immediately, even though the levels of aconitase and isocitrate-dehydrogenase in cell-free extracts of cells grown with glucose or citrate were approximately the same. No citratase was detected. These results agree with those of Davis (9) and suggest that the lag phase is related to the synthesis of a permeability system.

When penetration of radioactive citrate was measured, it was observed that internal radioactivity in uninduced bacteria remained much below the level of external radioactivity during a period of about 8 min. Addition of chloramphenicol had no effect on this behavior. On the other hand, citrate uptake by induced cells was

linear during the whole period of observation (Fig. 1). These results showed further evidence that the inducible system is involved in permeability.

Of the total radioactivity of the induced cells, 97.7 to 100% was extracted by hot water; of this extract, 40 to 70% of the radioactivity was recovered as citrate. These results clearly indicate that there is indeed citrate accumulation in the cells.

Induction was completely prevented by chloramphenicol and *p*-fluorophenylalanine, but oxidation of either glucose or citrate by induced bacteria was unaffected by either the antibiotic or the amino acid analogue. When uninduced cells were deprived of nitrogen by shaking them in a medium lacking a nitrogen source and then recovered and tested for their ability to metabolize citrate, no induction was observed at the end of 4 hr of incubation with citrate; however, glucose utilization and citrate oxidation by induced cells was not affected by such treatment. These results indicate that synthesis of protein is required before the cells can utilize citrate.

In a further experiment, *A. aerogenes* Ou $^-$ Arg $^-$ (22) was grown in either a glucose or a citrate medium; the cells were recovered and starved for 3 hr for arginine in the same basal medium. The cells were then centrifuged, washed, and tested for their ability to oxidize citrate in the presence or absence of arginine. Uninduced cells incubated with arginine showed the usual induction phase before they started oxidizing citrate. When no arginine was added, the cells

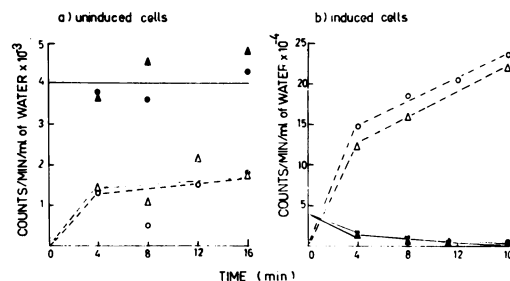


FIG. 1. Citrate penetration into uninduced and induced cells of *A. aerogenes* Ou $^-$ Arg $^-$. Cells (1.4 mg, dry weight) were incubated with citrate-1,5- ^{14}C . At intervals, samples were filtered through membrane filters and counted. Samples from the filtrate were also counted. Data are expressed as counts per minute per milliliter ($\times 10^3$ in Fig. 1a and $\times 10^4$ in Fig. 1b) of cell water or of extracellular fluid. Closed figures, external radioactivity; open figures, internal radioactivity; circles, no chloramphenicol added; triangles, cells incubated with 50 μg of chloramphenicol per ml.

showed only a weak and late induction, which may be explained by assuming that there is turnover of the cell proteins which provides arginine for the synthesis of the permeability system. Davis (9) reported that a tryptophan auxotroph was induced only in the presence of the required amino acid.

It has been stated that synthesis of ribonucleic acid (RNA), but not of deoxyribonucleic acid is a prerequisite for the synthesis of an inducible enzyme (15). Therefore, we investigated whether a uracil analogue (5-fluorouracil) would inhibit induction of the cells by citrate. 5-Fluorouracil was shown to be a strong inhibitor of growth, but it showed only a very slight effect on the induction process when added to uninduced "resting cells," in contrast to its effect on β -galactosidase induction (24).

These data may be interpreted by assuming either that fluorouracil cannot effectively compete with the endogenous uracil of the cell, or the 5-fluorouracil can substitute for uracil in the synthesis of a messenger RNA (mRNA) which directs the synthesis of the protein involved in citrate permeability. To gain more information on this point, *A. aerogenes* Ou^- was grown in a glucose medium; the cells were recovered, washed, and resuspended in fresh medium containing 0.1% Casamino Acids (Difco), but lacking uracil. The flasks were then shaken for 2 hr to decrease the intracellular level of uracil. The cells were then divided into four portions and incubated in flasks containing the basal medium plus: (i) 0.5% citrate; (ii) 0.5% citrate plus 100 μ g of uracil per ml; (iii) 0.5% citrate plus 100 μ g of fluorouracil per ml; and (iv) 100 μ g of uracil per ml. The flasks were shaken at 37 C for 3 hr, the cells were recovered by centrifugation and washed, and the respiratory activity on citrate was measured (Fig. 2). Cells incubated in the presence of citrate and uracil oxidized citrate without any lag phase. Cells incubated with either citrate or uracil alone did not oxidize citrate during the observation period, a result consistent with the hypothesis that synthesis of a specific mRNA is required to obtain synthesis of the transport system. Surprisingly, cells incubated in the presence of citrate and 5-fluorouracil showed some respiratory activity. This result may be explained by assuming that 5-fluorouracil substitutes for uracil in the synthesis of an mRNA which is less efficient than the normal one in directing the synthesis of the transport system or which directs the synthesis of an abnormal transport system.

To study the kinetics of the synthesis of the transport system under conditions in which citrate was not the limiting factor for growth,

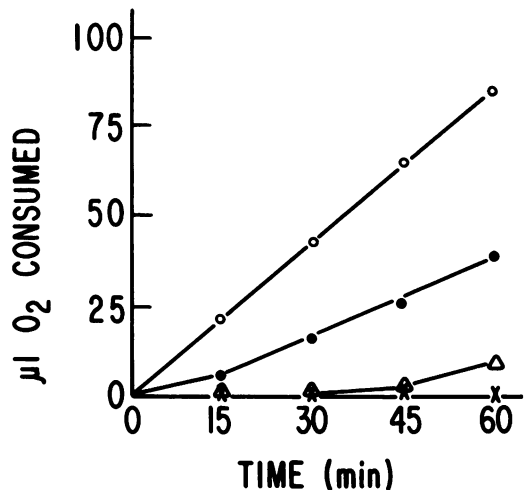


FIG. 2. Requirement of uracil for induction of citrate transport system. Cells (1.4 mg) cultivated under different conditions were incubated in Warburg flasks and oxygen uptake was recorded; substrate was 10 μ moles of citrate. Cells incubated in a medium containing citrate and uracil (O); cells incubated in a medium containing citrate and 5-fluorouracil (●); cells grown in a medium containing citrate only (Δ); cells incubated in a medium containing uracil only (\times).

several secondary carbon sources were tested. Glucose, succinate, and mannitol were the best carbon sources, but in all cases synthesis of the transport system was completely repressed. When peptone (Difco) was added to the medium, growth was the same in the presence or absence of citrate, and therefore peptone was added in further experiments. The general protocol was to inoculate a heavy cell suspension of glucose-grown cells into a peptone medium and to incubate it for 1 hr. At this time, the medium was divided into two portions; 0.5% citrate was added to one portion. At different times samples were withdrawn, their turbidity was measured, and chloramphenicol was added to a final concentration of 50 μ g/ml. The cells were recovered by centrifugation. The cells were washed with buffer containing chloramphenicol and resuspended in the same buffer. Finally, the turbidities of all samples were adjusted to the same value and the respiratory activity of the samples was measured. A differential plot drawn from the results obtained in one experiment is shown in Fig. 3. Induction followed the classical kinetics described by Jacob and Monod (15) for inducible systems, the system being synthesized as a constant fraction of the cell mass, except that the activity did not extrapolate back to the time of the addition of the inducer.

Specificity of the transport system. Uninduced

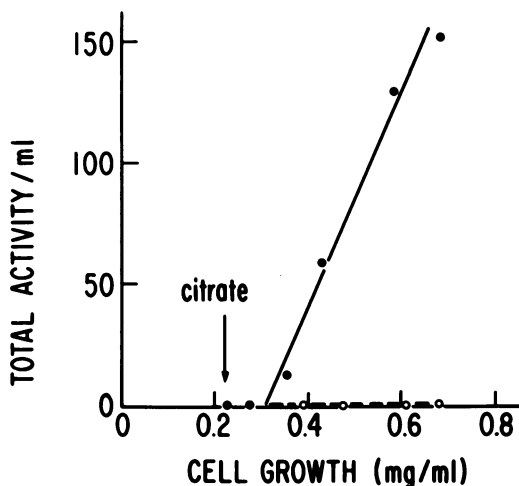


FIG. 3. Induction of the citrate transport system in *A. aerogenes* strain 1143 O_u^- . Cells grown in a peptone medium received (at arrow) 0.5% citrate; samples were removed at intervals, washed, and diluted to contain 1.4 mg (dry weight) of cells. Respiratory activity with citrate as substrate was measured by a protocol similar to the one described for Fig. 2. Total activity is expressed as microliters of O_2 per milliliter of culture medium. Citrate added to the growth medium (\bullet); no citrate added to the medium (\circ).

cells oxidized glucose and oxaloacetate without any lag phase, but they oxidized citrate, isocitrate, and cis-aconitate after a lag phase. Induced cells oxidized citrate without any lag phase, their behavior towards the other substrates being the same as that of uninduced cells. These results show that the citrate transport system is highly specific, not acting even on very similar compounds.

Saturation of the transport system. The increase in rate of penetration by citrate was not directly proportional to the concentration of citrate (Fig. 4). This type of kinetics has been described for most carrier-mediated permeability systems (16).

Effect of some inhibitors. It has been shown that uranyl ion blocks the transport of sugars in yeasts (26) and of amino acids in bacteria (6). This ion forms a complex with phosphate and carboxyl groups but not with sulfhydryl groups. The effect of uranyl ion on respiration was tested at pH 4.0 (Fig. 5). These results are expressed as percentage of inhibition versus the logarithm of the concentration of uranyl ion. The inhibition of glucose oxidation was biphasic, whereas the inhibition of citrate oxidation was monophasic. In addition, glucose oxidation was more sensitive than citrate oxidation to uranyl poisoning. These differences can be explained by assuming that

uranyl ion blocks two different types of ligands necessary for glucose penetration, only one of them being required for citrate penetration.

Treatment of the cells with 10^{-3} M *N*-ethyl maleimide (NEM) and 10^{-3} M dinitrofluorobenzene (DNFB) for 10 min at 37 C completely suppressed the respiratory activity of whole cells.

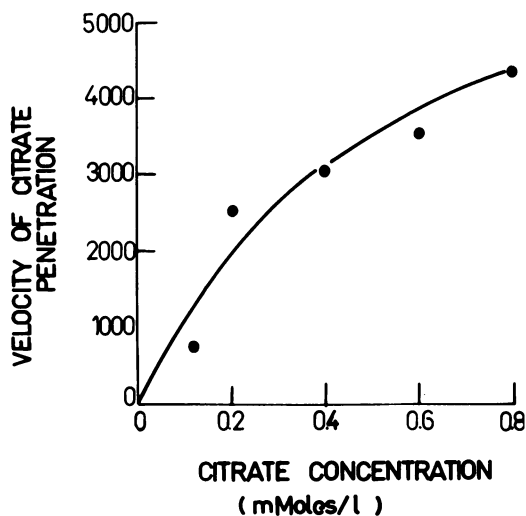


FIG. 4. Saturation curve of citrate permeability system in *A. aerogenes* $O_u^- Arg^-$. Cells (1.4 mg) were incubated with different concentrations of citrate-1,5- ^{14}C . After 3 min, samples of cells were filtered through membrane filters and the radioactivity was counted. Velocity of penetration is expressed as counts per minute per milliliter of cells at the end of 3 min.

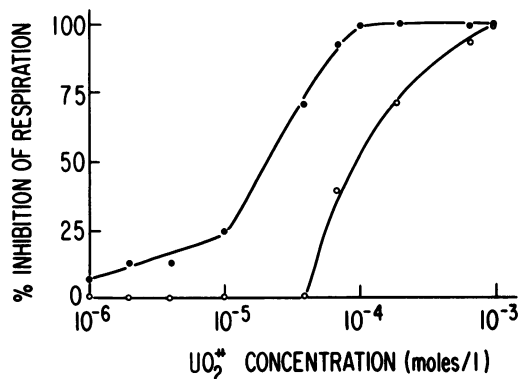


FIG. 5. Effect of uranyl nitrate on citrate penetration in *A. aerogenes* strain 1143 O_u^- . Cells were grown in a synthetic medium with citrate as carbon source, washed, and suspended in distilled water, pH 4.0. Respiratory activity was measured by a protocol similar to that described for Fig. 2, except that no phosphate was added (pH 4.0), and different concentrations of uranyl nitrate were added to the flasks. Glucose as substrate (\bullet); citrate as substrate (\circ).

However, cell-free extracts prepared from the treated cells showed isocitric dehydrogenase activity. Citrate partially protected the cells from the inhibitory effect of NEM. This protection was small but highly reproducible (Table 1).

Cyanide, azide, and dinitrophenol (DNP) were also inhibitors of citrate penetration. Citrate oxidation measured by DCPIP reduction was completely inhibited by 10^{-2} M KCN, but glucose oxidation occurred in the presence of cyanide (Fig. 6). It was also found that isocitrate dehydrogenase, aconitase, and citrate oxidation determined with DCPIP and phenazine methosulfate measured in cell-free extracts were not affected at all by 10^{-2} M KCN. These results are interpreted to mean that cyanide completely blocks citrate penetration into the cell. Effects of 10^{-3} M DNP, azide, and cyanide on 14 C-citrate uptake are shown in Table 2. All three compounds inhibited citrate penetration into the cell. In a further experiment, the effect of 10^{-2} M and 10^{-3} M KCN on the kinetics of citrate penetration was measured (Fig. 7). In our tests, 10^{-3} M KCN reduced the

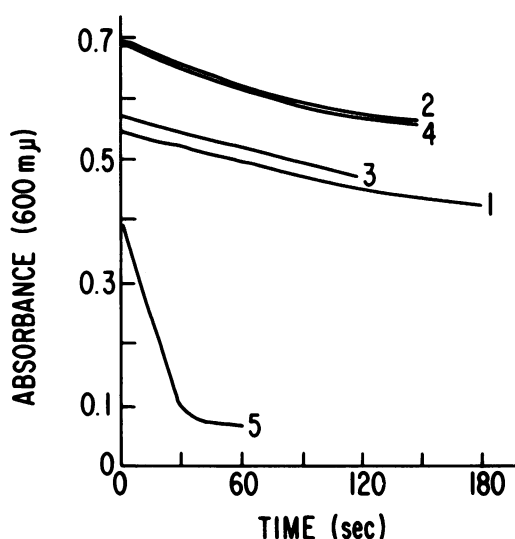


FIG. 6. Effect of KCN on citrate and glucose oxidation by whole cells of *A. aerogenes* strain 1143 Ou^- . Cells 280 μ g, dry weight) were incubated with 0.076 μ moles of DCPIP, 0.3 mg of phenazine methosulfate, 20 μ moles of either glucose or citrate, 0.033 μ moles of KCN, and 0.15 μ moles of potassium phosphate; final volume 3.0 ml, pH 7.3. Changes in absorbance at 600 nm were measured with a Photovolt Varicord model 43 recorder coupled to a Beckman DU spectrophotometer. The figures represent the trace of the recorder. (1) Cells grown with citrate and no substrate added (endogenous); (2) cells grown with glucose and no substrate added (endogenous); (3) citrate-grown cells with citrate as substrate; (4) glucose-grown cells with citrate as substrate; (5) citrate-grown cells with glucose as substrate.

TABLE 1. Inhibition of respiration and of isocitric dehydrogenase activity^a

Inhibitor	O ₂	Respiration inhibition	Isocitric dehydrogenase	
			Activity ^b	Inhibition
None	4.1	%	4.2	%
10^{-3} M NEM	0.15	97	0.66	84
10^{-3} M NEM + citrate	0.41	90		
10^{-3} M DNFB	0	100	2.3	45

^a *A. aerogenes* strain Ou^- was grown in medium containing citrate as carbon source. The cells were recovered, washed, and resuspended in 0.05 M phosphate buffer (pH 7.3). The cells were then divided into four flasks; 1 mmole of NEM and 1 mmole of DNFB were added to two of the flasks. One of the other flasks received 0.5% citrate and, after 10 min, 1 mmole of NEM. The fourth flask did not receive any additions. After incubation of the flasks for 10 min at 37 C, excess NEM and DNFB were eliminated by addition of 2-mercaptoethanol and glycine, respectively. The cells were centrifuged and washed with buffer. Respiratory activity of the treated cells was measured as previously described with citrate as substrate. Cell-free extracts of samples of the treated cells were obtained by ultrasonic oscillation, and isocitric dehydrogenase was measured in the supernatant fluid.

^b Change in absorbance brought about by 1 mg of protein in 1 min.

TABLE 2. Inhibition of the uptake of citrate by *A. aerogenes*^a

Inhibitor	Citrate uptake ^b	Inhibition
		%
None	22,210	
KCN	2,790	87
NaN_3	3,370	85
DNP	2,750	87

^a A 1.4-mg amount of citrate-grown cells of *A. aerogenes* strain 1143 Ou^- were incubated in a final volume of 4.9 ml of phosphate buffer (pH 7.3) with 5 μ moles of inhibitors for 15 min; 2 μ moles of citrate-1,5- ^{14}C was added and 1-ml samples were removed after 5 min of incubation at room temperature. The samples were filtered through membrane filters and washed. Radioactivity in the cells was then measured.

^b Expressed as counts per minute per milliliter of cells.

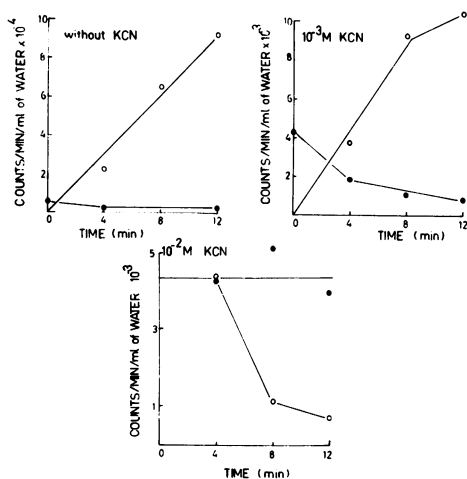


FIG. 7. Effect of KCN on citrate-1,5- ^{14}C penetration into *A. aerogenes*. Citrate-grown cells (1.4 mg) were incubated in a final volume of 4.9 ml of 0.05 M phosphate buffer (pH 7.3) with KCN at the indicated concentrations. After 10 min, 2 μmoles of citrate-1,5- ^{14}C in a volume of 0.1 ml was added, and 1-ml samples were removed at the indicated times. Cells were filtered, washed, dried, and counted. Samples of the filtrate were also dried and counted. Data are expressed as in Fig. 1. Notice the difference in the scale. External radioactivity (\bullet); internal radioactivity (\circ).

rate of citrate penetration to about 14% of the normal; however, there was still citrate accumulation. On the other hand, in the presence 10^{-2} M KCN, there was no citrate uptake and the level of intracellular radioactivity remained below the extracellular level. When uptake of citrate-1,5- ^{14}C was measured at 0 C, transport was completely suppressed during the period of measurement as judged by the fact that the internal radioactivity level remained lower than the external level of radioactivity (Fig. 8).

DISCUSSION

From our results, it may be concluded that the limiting step for the utilization of exogenous citrate by glucose-grown *A. aerogenes* is the synthesis of a specific permeability system. This conclusion agrees with existing data (9).

Evidence for protein as a component of the permeability systems for citrate in bacteria involves the inhibition of their synthesis by chloramphenicol (7, 14), by *p*-fluorophenylalanine (4), and by amino acid starvation in an auxotrophic mutant (9). By using all these criteria and also by nitrogen starvation, it was shown that the synthesis of a protein is required before the noninduced cell can utilize citrate. These data are in agreement

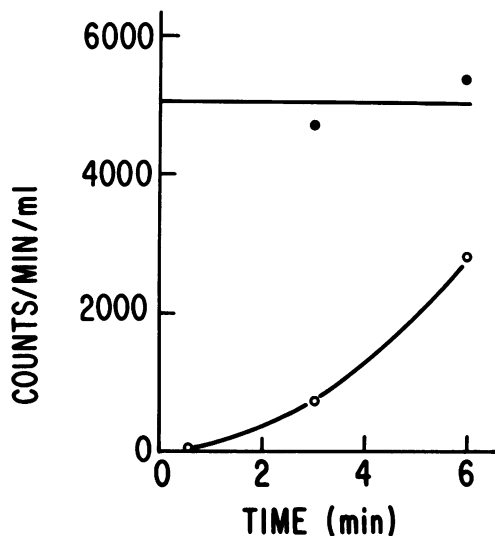


FIG. 8. Citrate penetration into induced *A. aerogenes* strain 1143 Ou^- at 0 C. Cells (1.4 mg) were incubated in an ice bath with citrate-1,5- ^{14}C , and penetration was measured as described in Materials and Methods and in the legend to Fig. 1. External radioactivity (\bullet); internal radioactivity (\circ).

with the general idea that proteins have a key role in permeability, as has been shown in bacterial systems (12, 25) and also in animal cells (1, 2, 11, 22; D. L. Oxender and B. Whitmore, *Federation Proc.*, p. 592, 1966).

As shown by the uracil requirement for induction in strain *A. aerogenes* strain 1143 Ou^- , the formation of the citrate transport system requires the previous synthesis of a specific messenger RNA. The induction of the citrate-transport system follows similar, if not the same, kinetics as those described for β -galactoside permease (18) and which are followed by most inducible systems (15). Synthesis of the citrate-transport system is inhibited by several carbon sources, which means that it is also subject to catabolite repression (23).

The results obtained with NEM demonstrate the involvement of SH groups in citrate permeability. These sulfhydryl groups are partially protected by citrate. Similar results have been obtained by Fox and Kennedy (12) and Fox et al. (13) for β -galactoside penetration in *E. coli*.

It has been generally considered that the energy required for "permease" activity (17) is involved in accumulation and not in transport itself (12, 18). The results obtained when the effect of KCN on citrate penetration was measured suggest that this may not be a general rule, because citrate penetration was suppressed by 10^{-2} M (Fig. 6 and 7). It is interesting to observe that

10^{-3} M cyanide, in contrast to 10^{-2} M cyanide, did not completely inhibit citrate penetration.

The two-step inhibition curve shown by uranyl nitrate and the fact that a higher concentration is required to inhibit citrate uptake than glucose uptake show that the mechanisms of transport of the two substances are different.

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