Citrate transport in *Salmonella typhimurium*: studies with 2-fluoro-L-*erythro*-citrate as a substrate

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The citrate analogue, 2-fluoro-L-*erythro*-[3,4,5,6-^{14}C]citrate was synthesized as a probe for the citrate transport system of *Salmonella typhimurium*. This analogue was actively transported by an inducible energy-dependent transport system with high affinity for fluorocitrate (K_m = 3.3 μM), and this transport system was inhibited competitively by citrate and isocitrate. Fluorocitrate was shown to be a competitive inhibitor of the citrate-binding protein (C protein) of this organism (K_i = 4–5 μM). Analogue resistant mutants were simultaneously defective in fluorocitrate transport as well as the C protein and the affected allele, *tctC*, was located at 59 units on the *S. typhimurium* chromosome map. These *tctC* mutants were shown to be specifically defective in K^+-dependent fluorocitrate transport but still retained another system capable of transporting fluorocitrate in the presence of both Na^+ and K^+.

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Le 2-fluoro-L-*érythro*-[3,4,5,6-^{14}C]citrate, analogue du citrate a été synthétisé et utilisé comme sonde pour l'étude du système de transport du citrate chez *Salmonella typhimurium*. Cet analogue est soumis à un transport actif par un système inductible dépendant de l'énergie qui présente une forte affinité pour le fluorocitrate (K_m = 3.3 μM) et qui est inhibé compétitivement par le citrate et l'isocitrate. La protéine affine pour le citrate (protéine C) de cet organisme est compétitivement inhibée par le fluorocitrate (K_i = 4–5 μM) Des mutants résistants à cet analogue sont simultanément incapables de transporter le fluorocitrate et dépourvus de protéine C; l'allèle affecté, *tctC*, est localisé à 59 unités sur la carte chromosomique de *S. typhimurium*. Ces mutants *tctC* ont perdu spécifiquement le transport du fluorocitrate dépendant des ions K^+, mais ont toujours conservé un autre système capable de transporter le fluorocitrate en présence simultanée des deux ions Na^+ et K^+.

Introduction

The transport of Krebs cycle tricarboxylates in microorganisms has not been as thoroughly explored as that of other systems (1). Some of the difficulties encountered have been due to both the rapid metabolism of tricarboxylate intermediates, as well as the inherent complications of multiple components or systems. For example, the transport of C_4 dicarboxylates in *Escherichia coli* is mediated by a system comprising at least three components (2–4), one of which may exist in at least two forms (5). With respect to L-aspartate, this system also overlaps with the C_5-dicarboxylate transport system (2, 6). The multiplicity of transport systems has apparently hampered the complete elucidation of C_4-dicarboxylate transport in *Bacillus subtilis* (7–10). Citrate transport in *Salmonella typhimurium* appears to be a complicated system as well. Genetic and biochemical evidence led to the suggestion that multiple systems mediate citrate transport in this organism (11–14). Recently, using aconitase (EC 4.2.1.3) mutants, FC-poisoned cells, and membrane vesicles, we described the general characteristics of an apparent citrate transport system in *S. typhimurium* (15). No kinetic evidence was obtained for other systems, although their existence was suspected.

The purpose of the present study was initially to document [^{14}C]FC as a useful nonmetabolizable probe of tricarboxylate transport in *S. typhimurium*. We also demonstrate that the *tctC* gene product, C protein, is requisite for FC incorporation into whole cells, and confirm the suggested existence of multiple transport systems or components.

Materials and methods

Bacteria and media

Strains of *S. typhimurium* used in this study are listed in Table 1. FC-resistant mutants (FC^r or *tctC*) were isolated as previously described (15, 16), and all mutants were automatically checked for their original auxotrophy. Cells were grown aerobically at 37°C in modified Davis

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ABBREVIATIONS: FC, fluorocitrate; [^{14}C]FC, 2-fluoro-L-*erythro*-[3,4,5,6-^{14}C]citric acid; C protein, citrate-binding protein; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TCA, trichloroacetic acid.

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TABLE 1. Bacterial strains

Strain	Genotype	Source and comments
LT2-Z		Prototroph, B. N. Ames, University of California, Berkeley, CA
TA3311	*cpd-401*	B. N. Ames
SR305	HfrA *hisD23 gal-50*	K. E. Sanderson, University of Calgary, Alta.
S16600	*argH95*	K. E. Sanderson
S16601	*argH95 tctC*	This study by spontaneous resistance of S16600 to FC
SU453	*hisF1009 trpB2 metA22 xyl-1 strA201*	K. E. Sanderson
KS191	*hisF1009 trpB2 metA22 xyl-1 strA201 tctC*	This study by spontaneous resistance of SU453 to FC
SA535	HfrK5 *serA13 rfa-3058*	K. E. Sanderson
SA640	HfrK2-2 *serA150*	K. E. Sanderson

minimal medium which contained K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; Na_2HPO_4, 8.65 g; $NaH_2PO_4 \cdot H_2O$, 4.57 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.97 g; distilled water, 1 L. Carbon sources were added separately at a final concentration of 10–20 m*M*. Where necessary, amino acid supplements were at 20 μg/mL.

Chemicals

L-[U-^{14}C]Malate and [1,5-^{14}C]citrate were purchased from Amersham Corporation (Mississauga, Ont.). Pig heart malic dehydrogenase (EC 1.1.1.37) and citrate synthase (EC 4.1.3.7)were from Sigma Chemicals (St. Louis, MO). CCCP and DL-FC were products of Calbiochem (La Jolla, CA).

Synthetic methods

Sodium fluoroacetate, barium fluoroacetate, fluoroacetic acid, fluoroacetyl chloride, and fluoroacetic anhydride were prepared as previously described (17). Fluoroacetyl-coenzyme A was prepared using the following modification of the method of Fanshier *et al.* (18): in order to give quantitative thiolesterification of coenzyme A the buffer concentration was increased 10-fold and a large excess of fluoroacetic anhydride was used. A nitroprusside spot test was used to monitor the disappearance of coenzyme A.

The enzymatic synthesis of [^{14}C]FC was accomplished by the following modifications to the method of Oehr and Willecke (19), with at least a 65% yield. The crude mixture was purified by a combination of ion-exchange chromatography, preparative thin-layer chromatography, and high voltage electrophoresis. The mixture was first treated batchwise with an excess of Dowex 50 (H^+), and the filtrate neutralized with saturated $(NH_4)_2CO_3$, mixed with 5 g activated charcoal for 30 min, then filtered. The filtrate was concentrated to dryness under reduced pressure. The residue was taken up in 0.2 mL water, streaked onto a single 0.5-mm cellulose thin-layer plate, and then dried and electrophoresed in 0.1 *M* formic acid – 0.1 *M* ammonium formate, pH 2.8. This procedure both desalted the mixture and also considerably sharpened the band at the origin. The plates were then chromatographed in the same dimension in ethyl ether – formic acid – H_2O (7:2:1 v/v). The products which separated into two well-defined bands were autoradiographically identified as FC and unreacted malate. These were eluted with water, concentrated, and the malate saved for successive syntheses.

Transport assays

Cells were induced for citrate transport by overnight growth in 50 mL modified Davis minimal salts medium containing 15 m*M* citrate as the carbon source. The cultures were grown aerobically at 37°C in 250-mL flasks agitated by rotation at 250 rpm. Cells were harvested at a culture density (A_{650}) from 1.0 to 2.0 by centrifugation and washed 3 times with unsupplemented minimal medium or uptake buffer. FC transport was measured by the usual membrane filtration technique previously described (2). [^{14}C]FC was always added as the free acid at very low concentrations to prevent the salt from affecting transport. Kinetic data were calculated from filtration data obtained from 30-s sampling intervals up to 2 min. Data obtained this way were analyzed by computer program (MICMEN) designed to provide regression analysis, standard deviation, percent error, K_m, and V_{max}. All automatic pipettors were previously calibrated with radiolabelled solutions. Transport rates are expressed on a per milligram dry weight of cells basis.

In vivo *[^{14}C]FC metabolism*

[^{14}C]FC was incubated with whole cells, and the filtered cells lysed, chromatographed, and submitted to autoradiography as previously described for [^{14}C]citrate (1).

Competitive inhibition of [^{14}C]citrate binding

The C protein was purified as recently described (16). Binding studies were carried out by membrane filtration in the presence of variable concentrations of [1,5-^{14}C]citrate or FC as also described (16). Citrate binding is expressed as a function of total citrate added, and approximate K_d values for citrate and K_i values for FC were computed from these data.

Genetic studies

Interrupted conjugation experiments were performed by the method of Sanderson *et al.* (20).

Results

Transport and metabolism of [^{14}C]FC

Wild-type strains of citrate-grown *S. typhimurium* accumulated [^{14}C]FC rapidly, but the extent of uptake varied and was dependent on the strains used. In no instance was [^{14}C]FC metabolized since no label was either lost as $^{14}CO_2$ or appeared in TCA-precipitable material (Fig. 1), as opposed to the rapid metabolism observed during [^{14}C]citrate uptake (15). The [^{14}C]FC intracellular pool thus formed was stable for at least 40 min and was not detectably modified (Fig. 1, inset). A closer inspection of the [^{14}C]FC transport

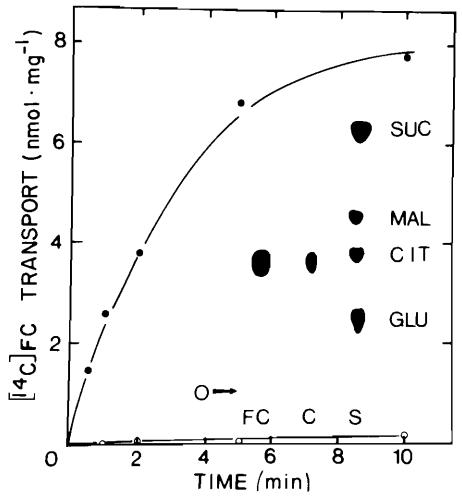

FIG. 1. Transport of [^{14}C]FC in *S. typhimurium* S16600. Citrate induced cells were incubated with 10 m*M* glucose for 10 min before the addition of 10^{-5} *M* [^{14}C]FC, 0.4 mCi/m*M* (●—●). Incorporation was measured by membrane filtration and total recovery (98%) determined after acidification of the reaction mixture at 10 min. Incorporation into TCA-precipitable material (○—○) was also measured by filtration. Inset: Thin-layer chromatography and autoradiography of the intracellular label collected after toluene treatment of cells (2). From left to right indicates authentic [^{14}C]FC (FC), intracellular material at 2 min (C), and standards (S). The origin is indicated (0).

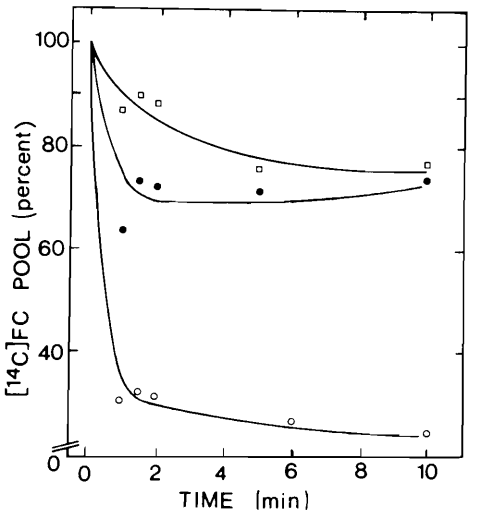

FIG. 2. Exchange of the fluorocitrate pool with unlabelled tricarboxylates. Citrate induced cells of *S. typhimurium* S16600 were prelabelled for 10 min as in Fig. 1, then aliquots were dispensed into separated vessels containing the unlabelled tricarboxylates citrate (○—○), DL-isocitrate (●—●), and *cis*-aconitate (□—□) to bring the final concentration to 1 m*M*. Aliquots were filtered at intervals thereafter.

TABLE 2. Competitive inhibition of [^{14}C]fluorocitrate transport by tricarboxylates[a]

Inhibitor (1 m*M*)	Inhibition (%)
None	0
Citrate	96.3
DL-Isocitrate	69.4
cis-Aconitate	12.2
trans-Aconitate	−8.1[b]
Tricarballylate	−28.9[b]

[a]Citrate-grown cells (S16600) were resuspended in Davis minimal salts containing both Na^+ (24 m*M*) and K^+ (24 m*M*). Transport was initiated by the addition of 10 μ*M* [^{14}C]FC (specific activity 0.4 mCi/m*M*; 1 Ci = 37 GBq) 10 min after energization with 10 m*M* glucose. Inhibitors were added simultaneously with [^{14}C]FC.

[b]Stimulation of [^{14}C]FC transport over the control.

process indicated that after an immediate binding reaction, the degree of which was found to be variable from experiment to experiment (Fig. 1), this probe was reproducibly transported linearly with time over short intervals and at a rate directly proportional to the cell concentration from 0.003 to 0.52 mg dry weight/mL. These conditions were then used to evaluate rate data.

[^{14}C]FC transport was shown to be competitively inhibited very effectively by citrate and isocitrate and somewhat poorly by *cis*-aconitate (Table 2). *trans*-Aconitate, tricarballyate, succinate, fumarate, and L-malate (data not shown) were found not to be effective inhibitors of [^{14}C]FC transport. Furthermore, only the tricarboxylates citrate, isocitrate, and *cis*-aconitate were able to elicit an efflux of [^{14}C]FC from preloaded cells (Fig. 2), confirming their relative interaction with this carrier. Inexplicably a small but significant proportion (~30%) of the intracellular label exchanged unusually slowly with tricarboxylates. Thus [^{14}C]FC fulfills the usual criteria as a radioactive nonmetabolizable transport analogue of citrate for *S. typhimurium*.

Energy requirement for active [^{14}C]FC transport

Effective citrate transport requires the addition of an exogenous energy source (15). This unusual requirement was equally true for [^{14}C]FC transport since the addition of various energy sources strongly stimulated [^{14}C]FC transport (Fig. 3); however, the degree of stimulation was found to vary depending on the energy source and bacterial strains used. For example, α-glycerolphosphate or D(−)-lactate effectively stimulated [^{14}C]citrate transport in SR305 (15), but were less effective with [^{14}C]FC transport in S16600. An unusual time course was observed during [^{14}C]FC transport in which the extrapolated uptake curve appeared to intersect the ordinate. Various experimental manipulations (lower temperatures, increased substrate concentrations or reduced cell concentrations, cells harvested from various growth stages, 5- to 10-s sampling times) failed to resolve these kinetics through the origin, and we could only conclude at this time that there must exist a high degree of undefined energy-

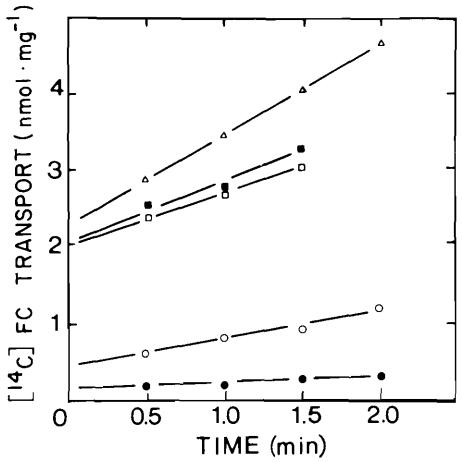

FIG. 3. Energy requirement for [^{14}C]FC transport in *S. typhimurium* S16600. Citrate induced cells were incubated for 10 min with 10 m*M* of the following energy donors: glucose (△—△), L(+)-lactate (■—■), pyruvate (□—□), succinate (○—○), glycerol, α-glycerolphosphate, or D(−)-lactate, glucose + 10^{-4} *M* CCCP, or control (●—●), prior to the addition of 10^{-5} *M* [^{14}C]FC.

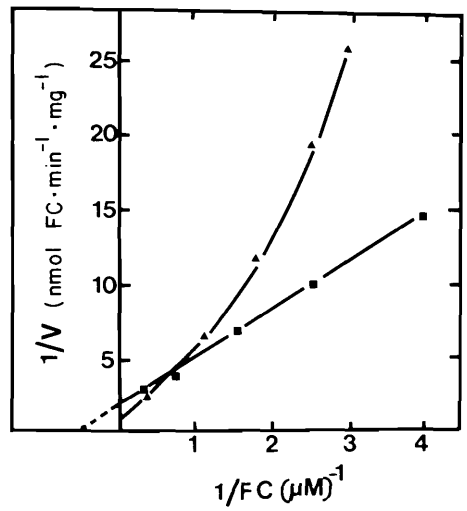

FIG. 4. Double reciprocal plot of the kinetics of [^{14}C]FC transport in strains of *S. typhimurium*. Citrate induced cells were incubated with variable [^{14}C]FC concentrations and rate data calculated from the linear rates up to 2 min by computer analysis (MICMEN). The strains shown are S16601 (FC^r, *tctC*) (▲—▲), and the wild-type S16600 (■—■). The kinetics for S16601 have not been extrapolated to a K_m.

dependent substrate binding to cells. The addition of CCCP, as well as other inhibitors, reduced [^{14}C]FC transport and the inexplicable binding as well to that of the nonspecific control (Fig. 3).

Kinetics of [^{14}C]FC transport in mutant strains

Previous [^{14}C]citrate kinetic studies in aconitase mutants only revealed a single transport component with a K_m of approximately 23 μ*M* (15). Using [^{14}C]-FC, kinetics of this kind could only be seen with the *cpd* mutant and not in wild-type strains which appeared to possess only a high affinity component (Fig. 4, Table 3) not observed previously in studies of citrate transport. Some variation was encountered in transport kinetics, especially for V_{max} values between different experiments (e.g., Fig. 4 and Table 3). However, affinities for wild-type strains were found to be from 2 to 5 μ*M*. Kinetics for FC^r (*tctC*) strains were very difficult to obtain as these strains could only be grown extremely slowly in citrate minimal media. Nevertheless, these anomalous kinetics do indicate progressively poorer transport at lower [^{14}C]FC concentrations, suggesting a defect in a high affinity component. These results also suggested the existence of at least two tricarboxylate transport components or systems of differing substrate affinities since [^{14}C]FC transport rates approached normal levels at higher substrate concentrations.

TABLE 3. Kinetic constants for [^{14}C]FC transport in *S. typhimurium* strains

Strain[a]	K_m[b] (m*M*)	V_{max} (nmol/min per milligram dry weight of cells)
LT2-Z (W$^+$)	2.88	1.13
S16600 (W$^+$)	3.27	1.30
TA3311 (*cpd*)	17.34	4.54

[a]Citrate-grown cells were washed and resuspended in Davis minimal salts containing both Na^+ (24 m*M*) and K^+ (24 m*M*). Transport was initiated by the addition of 10 μ*M* [^{14}C]FC (specific activity 0.4 mCi/m*M*) 10 min after energization with 10 m*M* glucose.

[b]Rate data were collected as described in Materials and methods, and K_m and V_{max} were calculated by computer analysis of the rate data.

Competitive inhibition of the citrate binding protein by FC

The C protein binds citrate with characteristic high affinity when measured by either equilibrium dialysis or membrane filtration (16). Because unusual technical difficulties are encountered with this protein in dialysis experiments, the technique of membrane filtration, which gives more consistent binding data, was used instead to demonstrate that FC is indeed a good competitive inhibitor of citrate binding to the C protein. By this method the K_d for citrate was found to be 2 μ*M* and the K_i for FC 5 μ*M*, a value which corresponds to the actual K_m for FC transport (Fig. 5).

Location of the tctC *allele*

All FC^r mutant colonies tested to date grow slowly on agar medium containing citrate, and selection for *tctC*$^+$ recombinants cannot be made on this medium owing to heavy background growth. FC^r mutants do not grow at all on isocitrate or *cis*-aconitate agar, and selection for *tctC*$^+$ recombinants was therefore made using these tricarboxylates. FC^r mutant KS191 was used as a recipient in interrupted mating experiments

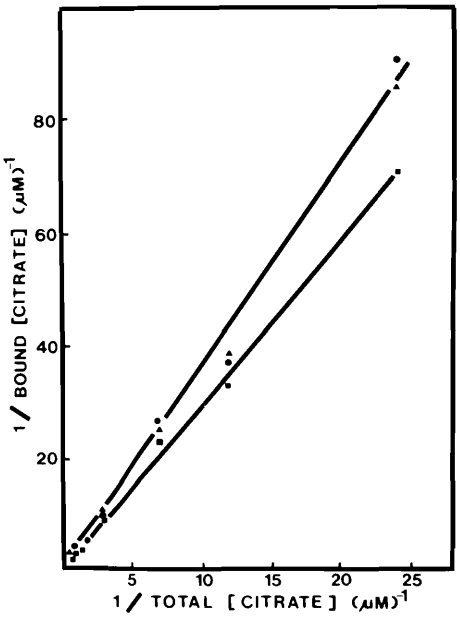

FIG. 5. Competitive inhibition of citrate binding to the C protein by FC. [1,5-^{14}C]Citrate binding was measured by membrane filtration (10), in the absence (■—■), as well as in the presence of either 10 μM Na^+-DL-FC (▲—▲) or 10 μM K^+-DL-FC (●—●). The C protein concentration was 14 μg/100 μL of filtered volume.

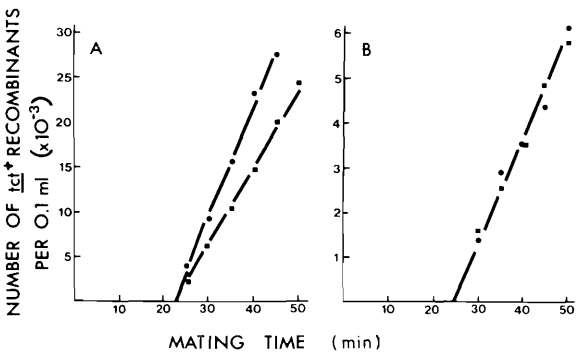

FIG. 6. Kinetics of formation of *tctC*$^+$ recombinants in FC^r mutant KS191. To 7 mL of broth 2 mL of an overnight culture of F$^-$ KS191 and 1 mL of a log Hfr culture were added. Aliquots were withdrawn at 5-min intervals, blended, diluted, and plated on minimal agar medium containing 20 μg/mL of appropriate amino acid supplements, 1 mg/mL streptomycin, and 0.1 M isocitrate (●—●) or *cis*-aconitate (■—■). (A) Hfr SA535; (B) Hfr SA640.

(20) to determine the location of the *tctC* allele. Crosses using Hfr SA535 as donor (point of origin at 66 min, clockwise transfer) (20) showed that *tctC*$^+$ enters at approximately 23 min after the initiation of mating (Fig. 6A). Hfr SA640 (origin at 117 min, counterclockwise transfer) transferred *tctC*$^+$ after approximately 25 min (Fig. 6B). These results place *tctC* between 89 and 92 min, i.e., at approximately 60

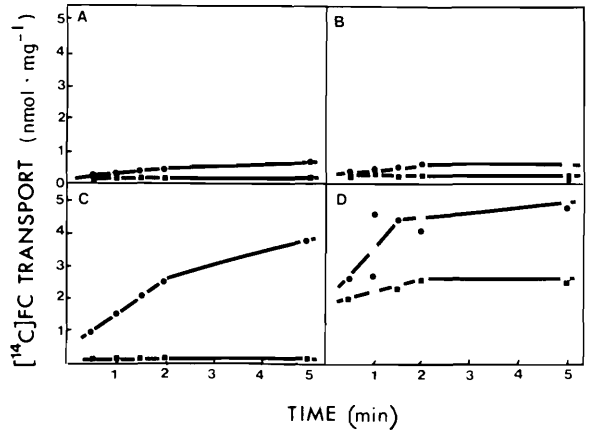

FIG. 7. Sodium and potassium requirements for [^{14}C]FC transport in strains of *S. typhimurium*. Citrate induced cells were washed 4 times in 10 mM trimethylammonium–HEPES at pH 7.0 and resuspended in the same buffer. Na^+ and K^+ as their chloride salts were added with 10 mM glucose 10 min before the addition of 10 μM [^{14}C]FC. (A) No salt added; (B) 10 mM Na^+; (C) 10 mM K^+, (D) 10 mM Na^+ + 10 mM K^+. The strains represented are S16601 (FC^r, *tctC*)$^-$ (■—■) and the wild-type S16600 (●—●).

units on the linkage map of *S. typhimurium* (21). Cotransduction studies with genetic markers in this region have located the gene more precisely at unit 59 on the chromosome in all FC^r mutants studied so far (J. M. Somers, G. D. Sweet, and W. W. Kay, manuscript in preparation).

Cation requirements for [^{14}C]FC transport in mutant strains

When [^{14}C]FC transport was studied as a function of the Na^+ or K^+ requirement in wild-type and FC^r strains, it was found that these ions were required at high concentrations not stoichiometric with [^{14}C]FC. Na^+ alone was essentially ineffective in stimulating [^{14}C]FC transport in the wild-type or FC^r strains (Fig. 7B); however, K^+ alone markedly stimulated [^{14}C]FC transport in the wild type (Fig. 7C). Surprisingly, both Na^+ and K^+ were required for effective [^{14}C]FC transport in FC^r strains and enhanced transport in the wild-type strains as well (Fig. 7D).

Discussion

Salmonella typhimurium transports citrate by systems apparently distinct from those for other TCA cycle intermediates (15) and this was also shown to be true for FC in this study. A detailed elucidation of multiple transport components and their interrelationships frequently requires diverse approaches which motivated us to use FC in this system. Thus the transport of citrate under nonmetabolizing conditions in aconitase mutants and of [^{14}C]FC have similarities: rapid uptake, energy dependencies, and identical substrate

specificities. However, there are apparent disimilarities, namely, greatly different affinities and specific ion requirements. Similar precedents have been set with other microorganisms. For instance, 3-fluoro-L-*erythro*-malate, a nonmetabolizable C_4-dicarboxylate analogue, was used to establish the existence of a high affinity C_4-dicarboxylate transport system in *Bacillus subtilis* (10), a system which had gone undetected by two independent groups using labelled substrates and appropriately blocked mutants (8, 9). Membrane vesicles also apparently revealed this high affinity dicarboxylate transport system (7). The transport of [^{14}C]FC has also been studied in *B. subtilis* (19) and preliminary results with FC resistant strains indicated the existence of possibly a second system which transported citrate slowly. The analogy to *S. typhimurium* is apparent: FC^r (*tctC*) strains are indeed transport defective but still retain a system which is both slow to induce and is expressed only in the presence of both Na^+ and K^+. This system has yet to be fully characterized but does not appear to be of the high affinity type (Fig. 7) and will support the growth of the strain slowly in Na^+–K^+ media. All wild-type strains so far tested exhibited unusually long (10-h) induction times for growth in citrate medium containing sodium, and this induction time was considerably lengthened in FC^r (*tctC*) derivatives, and the growth rate substantially reduced. However, in media without sodium, wild-type strains take even longer (10–20 h) to induce on citrate, and *tctC* strains do not grow at all. Taken together with the binding of FC to the C protein, and the absence of C protein in *tctC* mutants (16), these data confirm that *tctC* mutants are indeed structural gene mutants coding for the binding protein, as it would be most unlikely to require both Na^+ and K^+ for an induction process. Furthermore, even after induction or slow growth on citrate the high affinity kinetics were still not regained. The high level of FC binding exhibited by cells particularly in the presence of Na^+ and K^+ (Figs. 3 and 7D) raises the possibility that a unique Na^+–K^+ specific tricarboxylate binding site or component exists. If this proves to be so, it acts to facilitate transport as evidenced by the ability of strains bearing the *tctC* mutation to grow slowly on Na^+–K^+ containing media, but not at all in only K^+-containing media.

The data herein suggest simply that [^{14}C]FC acts as a probe to detect the activity of the tricarboxylate-specific C protein. The *in vivo* and *in vitro* affinities and substrate specificities are nearly identical and FC resistant mutants were both transport and binding protein defective. All mutants spontaneously resistant to FC so far map in the same region of the chromosome as well (J. M. Somers and W. W. Kay, unpublished results).

As we see it the data indicate the likelihood of three systems or components effecting tricarboxylate transport: a low affinity system kinetically exhibited by wild-type (15) and *cpd* mutants, a high affinity system described here for wild-type strains, and a residual Na^+–K^+-dependent system still apparent in FC^r (*tctC*) mutants. The interrelationships of these systems remain to be elucidated.

It has not escaped our attention that *cis*-aconitate does not compete effectively for citrate or fluorocitrate transport in whole cells nor does it compete for binding by the C protein. Yet *tctC* mutants described here and elsewhere (16), are unable to grow on *cis*-aconitate as a sole carbon source (Fig. 6). The possibility that the *tctC* region harbours a gene required for *cis*-aconitate transport is currently under investigation.

Other investigators have suggested the existence of as many as four tricarboxylate transport systems from data on mutant phenotypes as well as citrate oxidation and uptake studies in unblocked strains (11, 13). While it is difficult to rationalize their data with the studies reported herein primarily because of the complication of citrate transport due to further metabolism, it is tempting to speculate that TCT I corresponds to our high affinity transport system, and that TCT II, which apparently transports *cis*-aconitate, perhaps corresponds to our low affinity system. Another system, TCT IV, which was suggested to be specific for citrate, perhaps corresponds to the Na^+–K^+ system revealed here in the *tctC* mutants. Even yet another system, TCT III, which was reported to be induced by tricarballylate may well represent the membrane localized tricarballylate dehydrogenase (22). A combined genetic and biochemical analysis should elucidate these systems even further. To this end Imai and co-workers (12, 14) have mapped two mutants by conjugation at approximately 56 units and a third at 1 unit on the *S. typhimurium* map (21). One of these is close to our assignment of 59 units and a comparative study of all these strains is underway.

Acknowledgements

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