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SUCCINATE TRANSPORT IN *BACILLUS SUBTILIS*. DEPENDENCE ON INORGANIC ANIONS

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SUMMARY

Cations were generally ineffective in stimulating succinate transport in a succinate dehydrogenase mutant of *Bacillus subtilis* unless accompanied by polyvalent anions; phosphate and sulfate being particularly active. The K_m values for the phosphate or sulfate requirement were approx. 3 mM.

Biphasic kinetics were characteristic of both the succinate (K_m values 0.1 and 1 mM), and inorganic phosphate (K_m values 0.1 and 3 mM) transport system(s). The phosphate transport system(s) was repressed by high inorganic phosphate and a coordinate increase in the transport of phosphate, arsenate, and phosphate-stimulated succinate transport accompanied growth in low phosphate media.

A class of arsenate resistant mutants were simultaneously defective in the transport of arsenate, phosphate and succinate when cells were repressed for phosphate transport, however, the transport of these ions was regained in these mutants when grown in low phosphate media. Organic phosphate esters did not stimulate succinate transport in arsenate resistant mutants but were effective after growth in low phosphate media. Growth under phosphate limitation permitted the simultaneous regain of both phosphate and sulfate dependent succinate transport activities whereas sulfate limitation alone was ineffective.

Succinate was not transported by an anion exchange diffusion mechanism since phosphate efflux was low or absent during succinate transport.

The transport of C_4-dicarboxylates in *B. subtilis* is strongly stimulated by intracellular polyvalent anions. The absence of an anion permeability mechanism precludes succinate transport but partial escape from this restriction is mediated by the derepression of a phosphate transport system.

INTRODUCTION

Many bacteria utilize Krebs cycle carboxylic acids as sole sources of carbon and energy. However, the mechanism by which these metabolites are transported and

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.

subsequently accumulated has not been extensively investigated. Theoretically, these substrates could enter the cell either as the uncharged protonated form at an unphysiologically low pH, or more likely as an ionized species at a higher pH. In order to transport anionic organic metabolites the cells must exercise compensating mechanisms to prevent unnecessary energy expenditure in forming both electrogenic and osmolar gradients. Such mechanisms presumably consist of either the cotransport of neutralizing equivalents of cations or the countertransport of excess anions from the cell. The well established mitochondrial di- and tricarboxylic acid transport systems appear to operate basically on at least three anion exchange systems [1, 2], two of which would be unlikely to function in bacteria due to inevitable carbon loss. Alternatively, there is the possibility that the counter-flow ion could in fact be an inorganic anion as in the mitochondrial L-malate-phosphate exchange system.

In *Bacillus subtilis* the tricarboxylic acid cycle intermediates have been shown to be transported by three kinetically and genetically distinct systems which are specific for the C_4-, C_5- and C_6-tricarboxylic acids [3–5]. Competitive inhibition data has indicated that citrate [6] and the C_4-dicarboxylic acids [5] are most likely transported as anions. Recently it was demonstrated that citrate transport in an aconitase mutant of *B. subtilis* is markedly dependent on the presence of divalent cations and that these cations are cotransported as a complex to preserve electroneutrality [7].

Succinate transport in *B. subtilis* has very unusual ion requirements relative to other metabolite transport systems in bacteria; that is that polyvalent anions are predominantly required for effective transport, but the anion requirement is not specific. We decided to investigate further the nature of this requirement since it has important implications regarding the mechanism of succinate transport and the interaction of this transport system with other anion and cation transport systems. We suggest that succinate transport is largely dependent upon a general inorganic anion equilibrating mechanism in the absence of which the tricarboxylic acid cycle ex-dicarboxylic acids cannot be transported.

EXPERIMENTAL

Organisms

The microorganisms used in this study were derivatives of the transformable strain *B. subtilis* 168, an indole auxotroph. *B. subtilis* 1Aa22, is a succinate dehydrogenase-deficient (*sdh*$^-$) mutant [8], and strains 4-2B and 4-5B are mutants derived from 1Aa22 which are resistant to 40 mM sodium arsenate. The succinate dehydrogenase mutants were routinely checked for purity by streaking on bromo-cresol purple indicator media [8]. The arsenate-resistant cells were also routinely checked for purity by streaking on low phosphate minimal agar and were routinely kept on nutrient agar containing 40 mM sodium arsenate. The isolation and characterization of strains 4-2B and 4-5B are described below. Stock cultures were kept either lyophilized or frozen in a medium containing 10 % glycerol, 0.1 % peptone and 20 mM L-malate.

Media and culture methods

Cultures were routinely grown on a New Brunswick gyratory shaker at 37 C in a minimal salts medium [9], to which sterile 0.1 % peptone and 20 mM L-malate were added separately. The above medium was modified by the replacement of inorganic

phosphate by 0.1 M Tris · HCl (pH 7.0) and either 5 mM α-glycerophosphate or 5 mM inorganic phosphate to obtain phosphate-limited cells. Sulfate-starved cells were obtained by growing cells in minimal medium in which $MgSO_4$ was replaced with $MgCl_2$, $(NH_4)_2SO_4$, with NH_4NO_3 and 0.2 mM L-cysteine. Cells were routinely harvested from mid-exponential phase, washed three times with large volumes of either cold minimal medium or the appropriate phosphate- or sulfate-deficient medium, or 10 mM Tris · HCl (pH 7.0) depending on the particular experiment. These cells were resuspended to 1.1 mg/ml (dry wt), and kept on ice for a short time prior to the transport experiments.

Isolation of arsenate-resistant mutants

Cells of *B. subtilis* 1Aa22, the succinate dehydrogenase mutants, were grown on 0.5 % peptone, harvested and spread on nutrient agar plates containing 40 mM sodium arsenate. Spontaneous mutants which arose at an approximate frequency of 10^{-7} were isolated after incubation at 37 °C for 48 h, and were subsequently purified several times on the same media and checked for the parental genetic markers. This isolation procedure is essentially similar to that described for *Escherichia coli* by Medveczky and Rosenberg [10] and Bennett and Malamy [11]. These strains were also routinely maintained on the same selection medium since they were found to undergo slow phenotypic reversion.

Transport assays

Measurements of the transport of radioactive 2,3-^{14}C-labeled succinate, ^{35}S-labeled sulfate, ^{74}As-labeled arsenate, or ^{32}P-labeled phosphate were performed using the rapid filtration technique previously described [5]. Briefly, reactions were carried out in 5 or 10 ml reaction mixtures containing 10^{-5} M radioactive substrates and 1.1 mg dry wt of cells per ml at 37 °C. Cells were intermittently filtered through membrane filters (0.45 μm pore size; Sartorius), washed with 4 ml of the same incubation medium, dissolved in 5 ml of scintillation fluid (PCS, Nuclear Chicago) and assayed for cellular radioactivity using a scintillation spectrometer (Nuclear Chicago, Mark II). Neither succinate, PO_4^{3-} nor AsO_4^{3-} were chemically altered during transport as determined by autoradiography [10].

For the determination of transport kinetic data initial rates of transport were calculated from the linear uptake measurements at 15-s intervals for 2 min. Routinely boiled cell controls were done in all succinate transport experiments to avoid anomalous nonspecific binding or complex formation. In no instance was significant background binding found to occur.

Chemicals

2,3-^{14}C-labeled succinate, ^{32}P-labeled orthophosphate, ^{35}S-labeled sulfate and ^{74}As-labeled arsenate were obtained from Amersham Searle Corp.

RESULTS

Ion requirement for succinate transport

Fig. 1A illustrates the general ion requirements for succinate transport in *B. subtilis* 1Aa22. Cells resuspended in distilled water were unable to transport

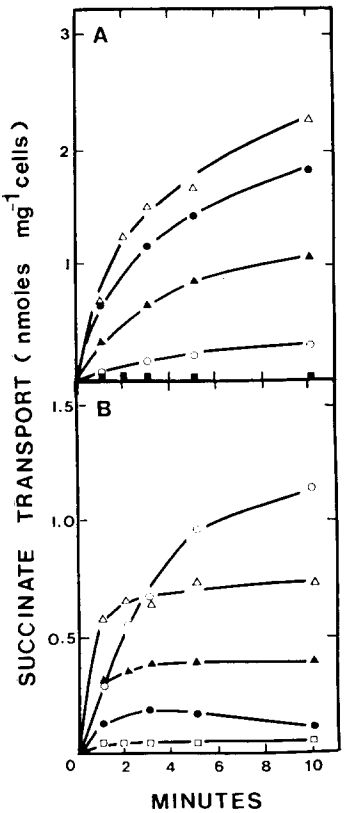

Fig. 1. Succinate transport into *B. subtilis* 1Aa22 in response to various cations and anions. Cells of the succinate dehydrogenase mutant previously induced for succinate by growth in the presence of 20 mM L-malate were washed (10 mM Tris · HCl, pH 7) and incubated in various ion combinations at pH 7.0 for 10 min at 37 °C prior to the addition of 2,3-^{14}C-labeled succinate (10^{-5} M, 0.05 μCi/ml). The cells were then quickly filtered and washed with the same buffer. The symbols in the graphs indicate the ion source present in the incubation and wash solutions. (A) Distilled water (■ – ■); 10 mM Tris · HCl (○ – ○); 10 mM K_2SO_4 (▲ – ▲); 50 mM K_2HPO_4 + KH_2PO_4 (● – ●) and 50 mM K_2SO_4 (△ – △). (B) 10 mM $Fe_2(SO_4)_3$ (□ – □); 10 mM $ZnSO_4$ (● – ●); 10 mM $FeSO_4$ (▲ – ▲); 10 mM Na_2MoO_4 (○ – ○) and 10 mM $MnSO_4$ (△ – △). Tris control (10 mM Tris · HCl) was the same as in Fig. 1A.

succinate and very low transport velocities were observed in 10 mM Tris · HCl buffer (pH 7.0). Although low, this concentration of Tris · HCl buffer was used as a control suspension media because it effectively prevented autolysis and provided a low enough background of succinate transport to compare the effects of added ions. Higher concentrations of Tris · HCl (20–100 mM) were inhibitory to dicarboxylate transport. Potassium phosphate or potassium sulfate stimulated both succinate transport and accumulation in a manner which was concentration dependent. At 50 mM both phosphate and sulfate salts were highly effective (Table I). Therefore, this system was not as ion-specific as has been frequently observed in various microorganisms for the transport of amino acids [12–17], carboxylic acids [18, 19] or sugars [20, 21].

TABLE I

SUCCINATE TRANSPORT IN THE PRESENCE OF VARIOUS CATIONS AND ANIONS

B. subtilis 1Aa22, a succinate dehydrogenase mutant was induced for succinate transport by growth on minimal medium and 20 mM L-malate. Cells were washed in 10 mM Tris·HCl buffer (pH 7.0) and resuspended (1.1 mg dry wt/ml) in the same buffer and preincubated with various salt solutions at 50 mM final concentration at pH 7.0 for 10 min. 2,3-^{14}C-labeled succinate (10^{-5} M, 0.05 μCi/ml) transport was then measured at various time intervals for 10 min. Data are expressed as relative initial rate of succinate transport and as relative final accumulation at 10 min.

Salt added	Succinate transport	
	Relative initial rate	Relative accumulation
None	1.0	1.0
KCl	1.3	1.0
NaCl	1.0	0.4
NH_4Cl	0.1	0.01
KNO_3	4.0	1.3
$K_2HPO_4 + KH_2PO_4$	10.7	12.6
$Na_2HPO_4 + NaH_2PO_4$	6.8	2.9
Na_2HAsO_4	6.8	2.4
K_2SO_4	5.1	3.2
Na_2SO_4	8.2	3.3
$(NH_4)_2SO_4$	4.3	0.7
Li_2SO_4	3.0	3.8
$MgSO_4$	11.3	2.8
$CuSO_4$	0.01	0.01
$K_2Cr_2O_7$	3.7	1.9
Potassium acetate	2.0	2.2
Potassium ascorbate	2.0	2.2
Potassium gluconate	2.5	2.1

The results of a comprehensive investigation of the ion requirements for succinate transport in *B. subtilis* 1Aa22 are shown in Fig. 1B and Tables I and II. Fig. 1B demonstrates that the initial rate of succinate transport and the final accumulation varied considerably with the ion source. The initial rate of succinate transport and the final level of accumulation were not always positively correlated. When present with di- or trivalent anions the monovalent cations, K^+, Na^+ and Li^+, stimulated both the rate of succinate transport and accumulation several-fold at 10 and 50 mM concentrations whereas little stimulation was observed when the chloride form of these ions, including Rb^+ and Cs^+, were used. The divalent cations were 2–3-fold more effective in stimulating the initial rate of succinate transport. However, the resulting final level of accumulation was invariably found to be lower than that observed with the monovalent cations. Again polyvalent anions were required since neither $MgCl_2$ nor $CaCl_2$ significantly stimulated succinate transport. Ammonium ion when present as the chloride was surprisingly markedly inhibitory, as was the heavy metal salt $CuSO_4$, which did not apparently complex with $Tris^+$ cation since it was equally inhibitory in 10^{-2}M Tris and 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer. Trimethylammonium chloride was without effect. The lack of a

TABLE II

SUCCINATE TRANSPORT IN THE PRESENCE OF VARIOUS CATIONS AND ANIONS

Experiments were carried out as described in Table I, except that cells were incubated with 10 mM concentration of the various ions.

Salt added	Succinate transport	
	Relative initial rate	Relative accumulation
KCl	2.3	2.7
CsCl	2.8	1.7
RbCl	2.5	1.6
KNO_3	1.0	1.6
NH_4NO_3	0.8	0.2
$KClO_3$	1.6	1.0
$CaCl_2$	1.2	0.8
$MgCl_2$	1.6	1.1
$Ca(NO_3)_2$	7.9	1.8
K_2SO_4	3.6	3.4
Na_2SO_4	3.4	4.7
$(NH_4)_2SO_4$	1.2	0.9
$CoSO_4$	13.6	2.1
Tris-SO_4	0.9	1.1
Tris-PO_4	0.7	0.7
TMA-SO_4*	0.10	1.1
TMA-Cl*	0.8	0.9
TMA-PO_4*	1.1	0.9

* TMA, trimethylammonium.

specific cation requirement has also been observed for succinate transport in membrane vesicles of *E. coli* [22].

Monovalent anions such as NO_3^- and Cl^- were not conducive to succinate accumulation. Nitrate (50 mM) stimulated the transport rate 3–4-fold but had little effect on the final accumulation. Chloride in the presence of Na^+, K^+, $Tris^+$ or trimethylammonium ions was ineffective. Chlorate was only slightly stimulatory. The presence of di- or trivalent anions (SO_4^{2-}, AsO_4^{3-}, PO_4^{3-}, $Cr_2O_7^{2-}$ and MoO_4^{2-}), however, were conducive to high transport rates and substrate accumulation, but not when present as the $Tris^+$ or trimethylammonium salts (Fig. 1, Tables I and II). $Cr_2O_7^{2-}$ stimulated succinate transport even at low levels (1 mM).

It is unlikely that osmolar effects would largely account for the very marked changes in succinate transport since both the chloride salts of various mono- and divalent cations and the Tris or trimethylammonium salts of various anions have no appreciable effect over a wide concentration range (Table II and unpublished results).

Dependence on anion concentration

When succinate transport was studied as a function of phosphate or sulfate concentration (Fig. 2), it was observed that approx. 20 mM potassium phosphate or 40 mM sodium sulfate were maximal for succinate uptake and that the half maximal concentration for these ions was each approx. 3 mM. These results indicate that a

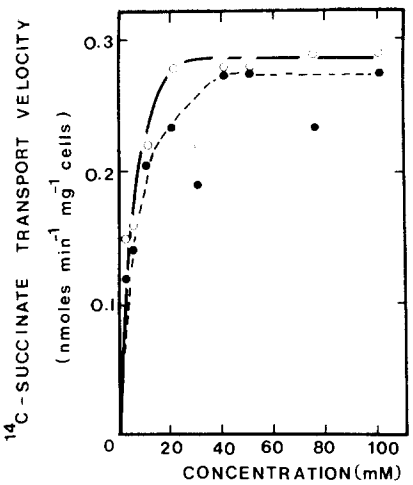

Fig. 2. Effect of phosphate or sulfate ion concentrations on succinate transport in *B. subtilis* 1Aa22. Cells were induced for succinate transport and subsequently washed twice with 10 mM Tris · HCl (pH 7.0) and resuspended in the same buffer. Na_2SO_4 or K_2HPO_4-KH_2PO_4 was added with 2,3-^{14}C-labeled succinate (10^{-5} M, 0.05 μCi/ml) at 0 min. Samples were filtered at 15-s intervals for 2 min, washed as described in Fig. 1 and the initial rates of succinate transport calculated. Salt additions: K_2HPO_4-KH_2PO_4 (○); Na_2SO_4 (●).

large difference exists between the kinetic parameters for succinate uptake (K_m values $1.0 \cdot 10^{-4}$M and 10^{-3}M) [4, 5] and the dependence on ion concentration and also demonstrate a lack of stoichiometry between succinate transport and the anion or cation requirement. It is therefore unlikely that the inorganic ion stimulation of succinate transport is simply due to the formation of a substrate-inorganic cation complex on the same transport carrier. The opposite effect has been demonstrated for magnesium ion and citrate transport in an aconitase mutant of this microorganism [7].

Coordinate derepression of phosphate and succinate transport

The phosphate transport system of *Bacillus cereus* has previously been shown to be derepressed 2–3-fold by inorganic phosphate starvation [24]. A similar situation was found in *B. subtilis*. Because of the instability of phosphate transport under these conditions, cells were grown in low phosphate medium (5 mM phosphate or 5 mM DL-α-glycerophosphate) similar to that previously devised for *E. coli* [11]. Fig. 3A demonstrates the increase in ^{32}P-labeled phosphate transport into cells of *B. subtilis* 1Aa22 grown under various conditions of phosphate limitation. Under these conditions, cells were able to transport phosphate at a 2–3-fold greater rate than cells grown in medium with excess phosphate. Similar results have been obtained for *B. cereus* [24]. A nearly identical coordinate increase was also observed for succinate transport (Fig. 3B). Phosphate stimulation of succinate transport appears to be related to the ability of these cells to take up and concentrate phosphate from the medium which likely excludes any non-specific external cell-surface phenomenon.

Kinetics of succinate transport in B. subtilis 1Aa22

Two different K_m values have been reported for dicarboxylic acid transport in

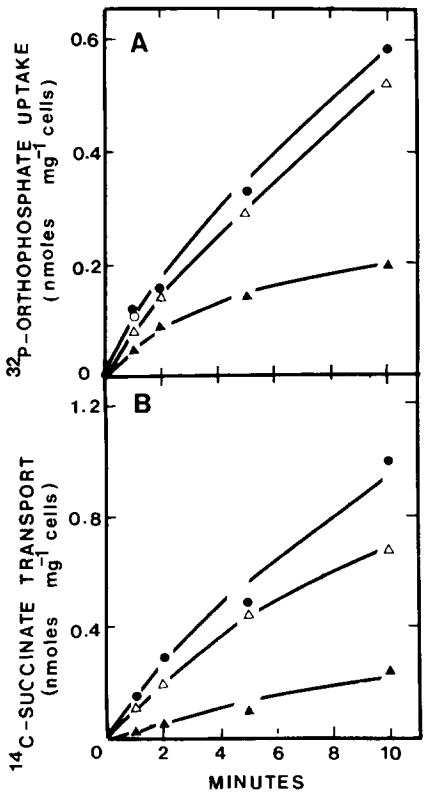

Fig. 3. Dependence of succinate transport on phosphate transport in *B. subtilis* 1Aa22. Cells were grown in phosphate sufficient or limiting phosphate medium, washed in 10 mM Tris · HCl (pH 7.0) medium and resuspended in 10 mM Tris · HCl (pH 7.0) for the measurement of ^{32}P-labeled phosphate transport and 2,3-^{14}C-labeled succinate transport. For the measurement of succinate transport 10 mM potassium phosphate was added with 2,3-^{14}C-labeled succinate at 0 min. Cells in the presence of 10^{-5} M ^{32}P-labeled phosphate (0.1 μCi/ml) (A) or 10^{-5} M 2,3-^{14}C-labeled succinate (0.05 μCi/ml) (B), were filtered at the indicated time intervals and washed with the same incubation media. (A) ^{32}P-labeled phosphate transport by cells grown on media containing 100 mM PO_4^{2-} (▲ ▲), 5 mM PO_4^{2-} (△ △), or 5 mM DL-α-glycerol-phosphate (● ●). (B) 2,3-^{14}C-labeled succinate transport by cells grown on some media coded as above.

B. subtilis [4, 5]. We redetermined these constants over a wider substrate concentration range. The kinetics for this system are in fact biphasic giving both K_m values previously reported (Fig. 4A). At higher concentrations (Fig. 4B) monophasic kinetics were observed which are nearly identical to that previously reported [4].

Isolation and characterization of arsenate resistant mutants

Twenty spontaneous arsenate resistant mutants of *B. subtilis* 1Aa22 were isolated as described in Experimental. Two mutants (4-2B and 4-5B) selected for further study were resistant to 40 mM concentrations of arsenate and were not dependent on arsenate for growth. These strains required high levels of phosphate (> 5 mM) for growth but grew well on low concentrations of DL-α-glycerophosphate

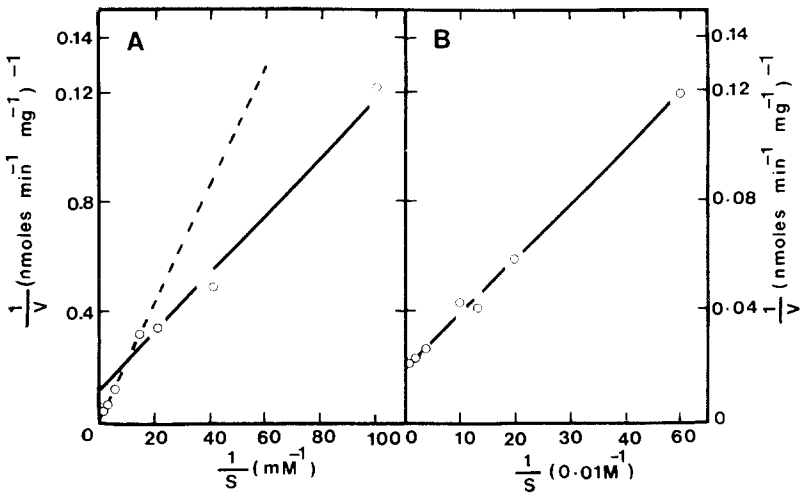

Fig. 4. Kinetics of succinate transport in *B. subtilis* 1Aa22 (*sdh*). Cells were grown in L-malate (20 mM) minimal media for induction. To establish initial rates, cells were filtered at 15-s intervals for 2 min. (A) indicates the biphasic concentration range and (B) demonstrates the kinetics at high substrate concentrations.

indicating no apparent defect in organic phosphate transport. Figs 5 A–C demonstrate that such mutants were not only defective in phosphate (Fig. 5A) and arsenate transport (Fig. 5C), but also were unable to effectively transport and accumulate succinate in the presence of phosphate as the stimulating anion (Fig. 5B). Strain 1Aa22 is unable to take up ^{74}As-labeled arsenate when grown on high phosphate medium (unpublished results), and can only be shown to do so when grown on limiting phosphate (Fig. 5C). Strain 4-5B is unable to incorporate arsenate under either of these growth conditions. These mutants showed no apparent defect in the uptake of aspartate, glutamate, proline, leucine or D-glucose when grown under phosphate, repressed or derepressed conditions (unpublished data), emphasizing the specificity of this effect.

Effect of medium composition and low temperature on phosphate-stimulated succinate transport in strains 1Aa22 and 4-5B

Incubation of strain 1Aa22 cells with 10 mM phosphate for 10 min prior to succinate transport resulted in the stimulation of succinate transport after growth on either high or limiting phosphate (Figs 6A and B). Little or no effect was observed on strain 4-5B grown on high phosphate (Fig. 6A), however, but a marked enhancement of succinate transport was observed with 4-5B cells grown on limiting phosphate (Fig. 6C). When 1Aa22 cells were chilled on ice with 10 mM phosphate no significant change in phosphate-stimulated succinate transport was observed (Fig. 6B), but chilling of mutant 4-5B under identical conditions restored succinate transport to levels approaching that exhibited by 1Aa22 cells (Fig. 6C). Nonspecific permeability at low temperatures due to "cold shock" has been reported [25, 26], and it is likely that 4-5B cells are made permeable to phosphate ions in this manner. These data

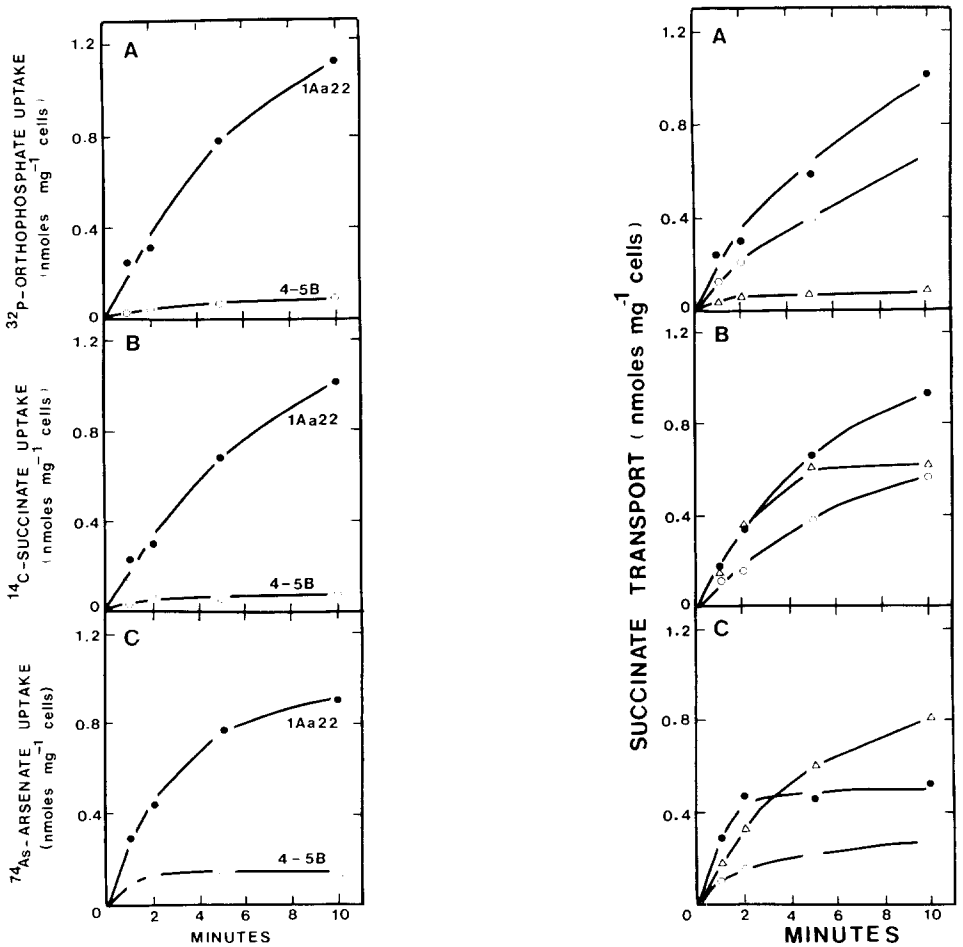

Fig. 5. Transport of phosphate (A), succinate (B), and arsenate (C) by *B. subtilis* 1Aa22 and its arsenate resistant derivative 4-5B. Cells were grown in high phosphate minimal medium for ^{32}P-labeled phosphate and 2,3-^{14}C-labeled succinate uptake and on 5 mM DL-α-glycerophosphate for ^{74}AS-labeled arsenate uptake (since cells grown on high phosphate did not show any detectable level of arsenate transport). Cells were washed three times with 10 mM Tris · HCl (pH 7) (A, C) or 10 mM Tris · HCl (pH 7.0) containing 10 mM potassium phosphate (B) and resuspended in the same buffer used for the wash. These cells were used for the transport assay of 10^{-5} M ^{32}P-labeled phosphate (0.1 μCi/ml) 2,3-^{14}C-labeled succinate (0.05 μCi/ml) and 10^{-5} M ^{74}As-labeled arsenate (0.1 μCi/ml), for 10 min. Strain 1Aa22 (●); strain 4-5B (○).

Fig. 6. Effect of phosphate preincubation on succinate transport in *B. subtilis* strains 1Aa22 and 4-5B. In A, cells were previously grown in high phosphate containing media but were washed three times in 10 mM Tris · HCl (pH 7.0). 2,3-^{14}C-labeled succinate transport was measured by the usual filtration method at 10^{-5} M. The isotope was added 10 min after preincubation with 10 mM PO_4^{2-} in strains 1Aa22 (● – ●) and 4-5B (△ – △) or simultaneously in 1Aa22 (○ – ○). Identical values were obtained with or without preincubation in 4-5B. In B and C, cells were first grown in low phosphate medium (5 mM PO_4^{2-}), 1Aa22 (B) or 4-5B (C). Cells were then incubated with 10 mM PO_4^{2-} for 10 min prior to the addition of 2,3-^{14}C-labeled succinate (● – ●) or both PO_4^{2-} and label were added together (○ – ○), or the cells were preincubated with 10 mM PO_4^{2-} at 0 °C for 60 min (△ – △).

TABLE III

EFFECT OF INORGANIC AND ORGANIC PHOSPHATES ON SUCCINATE TRANSPORT BY *B. SUBTILIS* 1Aa22 AND 4-5B

The succinate dehydrogenase mutant (1Aa22) and its arsenate-resistant derivative (4-5B) were grown on high phosphate (100 mM) and limiting phosphate (5 mM DL-α-glycerophosphate) media. 2,3-^{14}C-labeled succinate transport (10^{-5} M, 0.05 μCi ml) was then measured in response to the addition of various organic phosphate esters or inorganic phosphate present at 10 mM. Initial uptake rates are expressed in nmol · min^{-1} · mg^{-1} dry wt and accumulation at 10 min as nmol · mg^{-1} dry wt.

Growth medium	Phosphates tested	Succinate transport			
		1Aa22		4-5B	
		Initial rate (nmol·min·mg)	Uptake at 10 min (nmol/mg)	Initial rate (nmol·min·mg)	Uptake at 10 min (nmol·mg)
High PO_4^{2-}	None	0.065	0.189	0.025	0.033
	Acetylphosphate	0.194	1.004	0.035	0.058
	DL-α-Glycerophosphate	0.140	0.896	0.039	0.063
	Glucose-6-phosphate	0.270	0.896	0.037	0.044
	Orthophosphate	0.194	0.821	0.047	0.095
Limiting PO_4^{2-} (5 mM DL-α-GP)	None	0.029	0.057	0.020	0.040
	Acetylphosphate	0.096	0.468	0.090	0.300
	DL-α-Glycerophosphate	0.057	0.339	0.090	0.300
	Glucose-6-phosphate	0.143	0.841		
	Orthophosphate	0.086	0.512	0.140	0.525

suggest that the primary effect of these inorganic anions on succinate transport is manifested intracellularly by a mechanism that is only indirectly related to phosphate ion uptake.

Acetylphosphate, glucose 6-phosphate and α-glycerophosphate were all stimulatory to succinate uptake in strain 1Aa22, but not appreciably in 4-5B, when these cells were grown on a high phosphate medium (Table III). However, these same organic phosphate esters, as well as inorganic phosphate, were more stimulatory when the cells of 4-5B were previously grown under phosphate limiting conditions. These results and those described earlier (Fig. 6C) suggest that on phosphate limitation the system(s) capable of transporting both phosphate esters and inorganic phosphate derived from these esters can supply the required phosphate for succinate transport. These strains were capable of hydrolyzing organic phosphate esters since they were able to use each of the above organic phosphates as a sole phosphate source for growth. Extracts were also shown to contain a non-specific phosphomonesterase, and were able to release phosphate from DL-α-glycerophosphate (unpublished results).

Effect of sulfate and phosphate starvation on succinate transport in strains 1Aa22 and 4-5B

Succinate transport was not stimulated by various other polyvalent anions in mutant 4-5B when grown on high phosphate (Fig. 7), demonstrating a lack of specificity of the system responsible for the stimulation of dicarboxylate transport.

Figs 8 A–F describe the comparative effects of anions on succinate transport in strain 1Aa22 and the arsenate resistant derivative 4-5B under conditions of sulfate

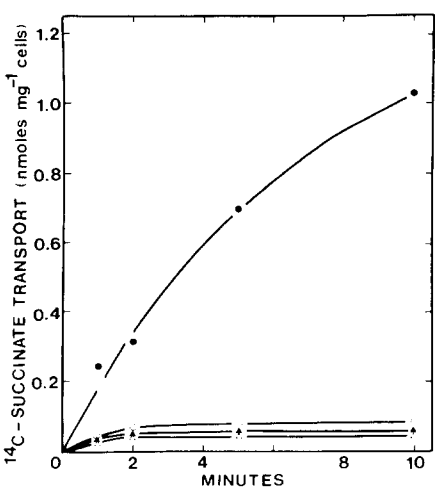

Fig. 7. Dependence of succinate transport by *B. subtilis* 1Aa22 and 4-5B cells on phosphate, sulfate and arsenate. Cells were grown on high phosphate minimal medium containing 20 mM L-malate, washed and resuspended in 10 mM Tris · HCl (pH 7.0). For measurement of 2,3-^{14}C-labeled succinate transport (0.05 μCi/ml in 10^{-5} M) 1Aa22 cells were preincubated for 10 min without any salt added. Tris control (○) or with 10 mM Na_2H PO_4 + NaH_2PO_4 (●), 4-5B cells were preincubated without any salt added. Tris control, with 10 mM and 50 mM Na_2HASO_4 (△), 10 mM and 50 mM Na_2HPO_4 + NaH_2PO_4 (▲), or 10 mM and 50 mM Na_2SO_4 (○)

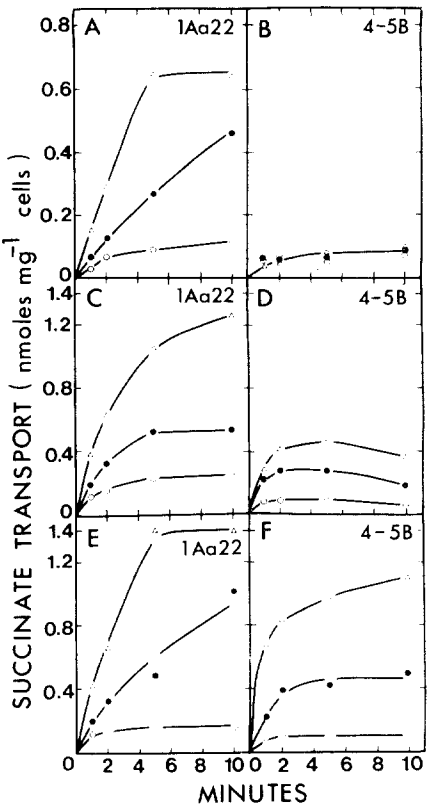

Fig. 8. Stimulation of succinate transport in sulfate and/or phosphate starved cells of *B. subtilis* 1Aa22 and 4-5B. Cells were grown either on limiting sulfate (0.2 mM cysteine) (A and B), or on limiting phosphate (5 mM DL-α-glycerophosphate) (C and D), or on limiting sulfate and limiting phosphate medium (E and F). 2,3-^{14}C-labeled succinate transport was measured by filtration at 10^{-5} M in the presence of various ions: A(1Aa22) and B (4-5B), Tris control (○ ○), 10 mM K_2SO_4 (● ●), and 50 mM K_2SO_4 (△ △); C(1Aa22) and D(4-5B) Tris control (○ ○); 10 mM Na_2HASO_4 (● ●); 50 mM Na_2HASO_4 (△ △); E (1Aa22) and F (4-5B) Tris Control (○ ○); 10 mM K_2SO_4 (● ●) and 50 mM K_2SO_4 (△ △).

and/or phosphate starvation. Sulfate starvation did not permit this ion to stimulate succinate transport in 4-5B (B), however, starvation for both sulfate and phosphate (Fig. 8F) permitted the regain of sulfate stimulated transport. Phosphate starvation only permitted the regain of phosphate or arsenate stimulated transport (Fig. 8D). These results suggest that the arsenate resistant strains grown on high phosphate media are defective in an inorganic anion transport system which profoundly affects dicarboxylate transport and that the concomitant defect with respect to succinate transport can be overcome through the regain of a specific phosphate transport system or another anion transport system. Repeated attempts to demonstrate $^{35}SO_4^{2-}$ transport in any of these cells were unsuccessful because of a technical difficulty due to poor sulfate retention, a situation previously reported for *Salmonella typhimurium* [27].

TABLE IV

KINETIC PARAMETERS FOR INORGANIC PHOSPHATE TRANSPORT IN *B. SUBTILIS* STRAINS 1Aa22 AND 4-5B

Cells were grown in either inorganic phosphate or DL-α-glycerophosphate containing media and were washed several times and resuspended in 10 mM Tris · HCl (pH 7.0) for transport experiments as described in Experimental Procedures.

Strain	Growth medium	K_m (mM)	V (nmol/min/mg cells)
1Aa22	100 mM PO_4^{2-}	0.1	1.3
		3.9	40.0
4-5B	100 mM PO_4^{2-}		
4-5B	5 mM DL-α-GP	0.1	2.5
		3.0	33.0

Kinetics of phosphate transport in strains 1Aa22 and 4-5B

Table IV lists the kinetic parameters obtained from a study of phosphate transport as a function of concentration in both phosphate sufficient and deficient cells by strains 1Aa22 and 4-5B. Strain 1Aa22 possesses two kinetically distinct systems for phosphate transport of high and low affinity. Under similar phosphate sufficient conditions no meaningful data could be obtained from strain 4-5B due primarily to the low uptake rates exhibited by this strain. Under phosphate limiting culture conditions, strain 4-5B regained the ability to transport phosphate by system(s) similar to that kinetically described for 1Aa22 grown in excess phosphate. Apparently

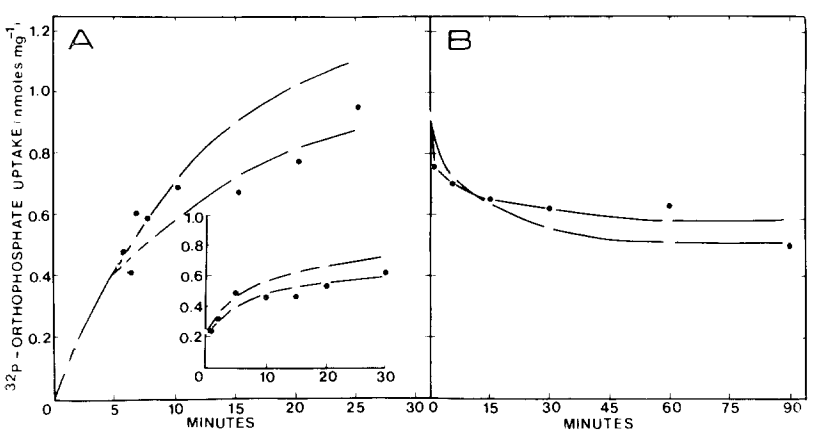

Fig. 9. A. Fate of ^{32}P-labeled orthophosphate in 1Aa22 cells grown on high phosphate minimal medium containing 20 mM L-malate. Cells were washed, resuspended and preincubated for 10 min in 10 mM Tris · HCl (pH 7.0) prior to incubation with ^{32}P-labeled orthophosphate at 10^{-5} M. At 5 min 10^{-2} M succinate was added and ^{32}P-labeled orthophosphate uptake measurements continued in cells without (○) and with succinate (●). Insert figure shows the fate of ^{32}P-labeled orthophosphate in cells labelled at 10^{-5} M for 5 min as above on addition of 10^{-4} M succinate. ^{32}P-labeled orthophosphate was measured in cells without (○) and with succinate (●). B. Fate of ^{32}P-labeled orthophosphate in cells labelled for 10 min at 1 mM final concentration. ^{32}P-labeled orthophosphate was measured in cells without (○) and with 20 mM succinate (●).

strains 4-5B and 4-2B are profoundly repressed for phosphate transport under growth conditions in excess phosphate. Strain 1Aa22 was unable to transport arsenate when grown in media containing high inorganic phosphate, but was active after phosphate limitation (Fig. 5C). Thus the phosphate-arsenate transport system appearing after phosphate limitation differs from systems operating after growth in the presence of excess inorganic phosphate by virtue of substrate specificity. Multiple phosphate transport systems have been reported also for *E. coli* [11] where an arsenate, and a phosphate-arsenate system have been lost through arsenate resistance.

The fate of intracellular inorganic phosphate in the presence of succinate

When cells of the succinate dehydrogenase mutant 1Aa22 were preloaded with inorganic phosphate at widely varying concentrations the addition of unlabelled succinate caused no apparent efflux of phosphate from the cells, neither during the uptake of phosphate (Fig. 9A), nor from phosphate loaded cells (Fig. 9B). Similar results were obtained with cells preloaded with succinate and washed or exposed to excess phosphate (unpublished data). These data demonstrate that the phosphate anion requirement is not due to the availability of counterflow anions and rule out the existence of an exchange-diffusion transport mechanism.

DISCUSSION

The maintenance of ionic equilibrium during the transport of charged metabolites, in particular Krebs cycle intermediates, in living cells could occur by several possible mechanisms. First, a cation or proton may be transported with an anionic form of metabolite stoichiometrically. This could occur either by the transport of the salt form of the metabolite by a specific transporter, by the cotransport of the cation by another site on the metabolite transporter in an anionic form or by the simultaneous operation of a specific cation transport system coordinated to preserve electroneutrality. Alternatively, ionic equilibrium could be maintained by an exchange-diffusion process in which excess anions are pumped out of the cell in response to transported dicarboxylate or tricarboxylate anions; a mechanism which could possibly supply the energetic requirements for active transport. As with cations this process could presumably occur either via the same carrier or by a separate site operating in response to the energy charge in the cell.

Several recent reports have indicated that Krebs cycle carboxylic acids are transported as anions in various bacterial cells [4, 5, 28, 29]. In *B. subtilis* citrate was shown to be stoichiometrically transported with magnesium ion by a system specific for divalent cations [7]. In *Aerobacter aerogenes* citrate transport was shown to have a specific requirement for either sodium or potassium in two separate conflicting reports [18, 19]. These cation specificities are apparently not adhered to during dicarboxylate transport in *B. subtilis* since all cations tested were stimulatory only when present with polyvalent anions, with the exception of ammonium, Tris, trimethylammonium and heavy metal ions. *E. coli* membrane vesicles do not appear to be particularly cation specific for succinate transport either [22], and succinate uptake in *Azotobacter vinelandii* as measured by succinate oxidation appears to be similar [29]. Although divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}) were consistently good stimulators of succinate transport in *B. subtilis*, there is always the possibility that

these ions may tend more to stabilize the microbial membrane rather than specifically participate in a transport process [31]. Furthermore, dicarboxylate competition data demonstrated that at least one ionized carboxyl was required for succinate transport [5]. If magnesium ions and succinate were transported as a complex then two substrate molecules would be required. Our data, however do not exclude the possibility that the cations are transported by separate specific cation transport systems to achieve electroneutrality [31, 32]. These studies are now in progress.

The requirement for polyvalent anions, for dicarboxylate transport is apparently unique and raises the fundamental question as to whether such anions are used in a counter-transport mechanism to maintain electroneutrality as demonstrated for the L-malate-phosphate exchange system in mitochondria [1, 2]. The coordinate increase in phosphate-stimulated succinate transport and phosphate transport by growth in low phosphate media and the isolation of mutants simultaneously defective in both phosphate and phosphate-stimulated succinate transport argue in favor of the requirement for inorganic anions to facilitate succinate transport. The regain of phosphate-stimulated succinate transport in mutant 4-5B by pre-incubation with excess phosphate at 0 °C and the stimulation of succinate transport by organic phosphates suggests that the anion requirement occurs intracellularly.

If anions are required intracellularly then their effect would not seem to be as specific as found for the mitochondrial system. That is either PO_4^{3-} or SO_4^{2-} etc. will suffice as the required ion. This indicates an unusual lack of specificity with regards to most microbial ion transporting systems. It is apparent from our data (Figs 9A and B) that these anions neither counter-transport dicarboxylate anions by the same transporter nor exit from the cell either by various specific ion transporters or by some general ion equilibrating system in response to dicarboxylate anions. However other anions such as HCO_3^-, a logical candidate as an end product of respiration, or perhaps even OH^- could possibly suffice in this regard.

Intracellular inorganic anions are perhaps required for stabilization of the succinate transport system but are not required as counter-transport ions. In this regard it would seem unlikely that anions would stabilize only dicarboxylate transport and not various amino acid or hexose transport systems. There is clearly a third alternative to explain the requirement for inorganic anions; that is that polyvalent inorganic anion transport is coordinated by cation transport, either cotransported by the same carrier or by specific carriers. This possibility is currently being investigated in various cation transport mutants. In this context it should be noted that intracellular phosphate greatly stimulated the accumulation of the cation dibenzyldimethylammonium chloride and also circumvented the inhibition of proline uptake by Na^+ in *E. coli* membrane vesicles [36].

Membrane vesicles of *B. subtilis* have been shown to accumulate carboxylic acids in a respiration-dependent manner [34], similar to that described for the transport of various metabolites by membrane vesicles of *E. coli* [35]. Recently it was demonstrated that *E. coli* vesicles formed a respiration-dependent membrane potential [36], which apparently could be directed toward active transport. If a common system (exterior positive and interior negative) were operating in *B. subtilis* during respiration it would underline the necessity for some charge compensating mechanism for dicarboxylate transport since transport as an ion would be against the respiration established electrogenic gradient. In this regard it is also of interest to speculate on the

nature of the mobilization of energy to effect active transport of dicarboxylates. Since almost all of the succinate exists as the dianion at pH 7.0, it is possible that if respiration sets up an electrochemical gradient by proton extrusion in *B. subtilis* then a proton may possibly accompany dicarboxylate transport at a carboxyl group effectively neutralizing it while simultaneously acting as the driving force for active transport. One carboxyl of the citrate molecule similarly is available for a similar activity [7].

The transport of inorganic phosphate in *B. subtilis* is also of basic interest. Clearly at least two transport systems operate with distinct kinetic parameters, which are derepressed by growth in low phosphate media. Arsenate-resistant mutants appear to be similar to their parent however, they are more strongly repressed by phosphate ions. A third transport system must also be operating since arsenate transport is only measurable in phosphate starved cells, in either parental or arsenate resistant cells. Also phosphate competitively inhibits arsenate uptake in both of these strains as has also been shown in *B. cereus* [24] and *E. coli* [11].

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