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TRANSPORT OF C_4-DICARBOXYLIC ACIDS IN *NEUROSPORA CRASSA*

L. WOLFINBARGER, Jr and W. W. KAY

Departments of Biochemistry and Surgery, University of Saskatchewan, Saskatoon, Saskatchewan S7N OWO, (Canada)

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SUMMARY

1. The transport of tricarboxylic acid cycle dicarboxylic acids by *Neurospora crassa* was induced 80-fold by growth on acetate whereas tricarboxylic acid enzymes were induced only 2–6-fold and glyoxylate cycle enzymes were induced 20-fold.

2. From the kinetics of uptake and competitive inhibition it was concluded that a single system was responsible for the transport of succinate, fumarate, L-malate and possibly α-ketoglutarate as well as a number of structural analogues. The system appears to be less specific than the known bacterial systems.

3. Succinate transport was strongly inhibited by various known inhibitors of energy metabolism — primarily by proton conductors. Transport was also severely inhibited by various sulfhydryl reagents.

4. Succinate, fumarate and L-malate uptake were identically pH dependent and evidence is presented which implicates the monoionic species as the molecular form of the dicarboxylic acid transported.

INTRODUCTION

The transport of polyanionic metabolites such as the tricarboxylic acid cycle intermediates across biological membranes represent a special situation in which highly polar negatively charged molecules permeate a relatively negatively charged hydrophobic barrier. It is of interest that the very early work on permeation in microorganisms involved such intermediates and led directly to the permease hypothesis[1–7]. Recently there has been renewed interest in this area of microbial transport resulting in the description of di- and tricarboxylic acid transport systems in *Escherichia coli*[8–12], *Bacillus subtilis*[13–17], *Azotobacter vinelandii*[18–19], *Streptococcus faecalis*[20], *Aerobacter aerogenes*[21–24] and *Pseudomonas*[25], which are apparently under genetic regulation; however, no description of similar systems in eucariotic microorganisms has yet appeared.

The transport of these intermediates in *Neurospora crassa* represents a potentially different situation where metabolite transport must occur at two levels: that of

Abbreviations: CCCP, carboxylcyanide-*m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HOQNO, hydroxyquinoline-*N*-oxide; PCMB, *p*-chloromercuribenzoate.

the cytoplasmic membrane for initial capture of the metabolite, and that of the mitochondrial membrane for its subsequent metabolism. At the latter level mechanisms of metabolite transport have been investigated widely in mammalian mitochondrial systems[26,27], but not at the level of the cytoplasmic membrane.

We felt that some system of permeation for tricarboxylic acid cycle intermediates must exist in *N. crassa* because of the previous isolation of a pyruvate carboxylase mutant which was auxotrophic for succinate[28]. This investigation represents initial studies which describe a transport system for dicarboxylic acids at the cytoplasmic membrane in germinating conidia of this organism.

METHODS AND MATERIALS

The wild type strain of *N. crassa* (SY4f8a) was used in these studies.

Preparation of cells for transport studies

Conidia were obtained by growth for 7 days on Vogels minimal media N (ref. 29) supplemented with 2% sucrose and 2% agar. The conidia were harvested with a sterile wire needle into sterile glass distilled water, filtered through cheese cloth and glass wool to remove mycelial fragments and stored in crushed ice (0 °C) until used. Dry weights of conidial suspensions were determined by filtering 5- or 10-ml aliquots onto tared nitrocellulose filters (Sartorius, 0.45 μm pore size) and drying to a constant dry weight.

For uptake and enzyme analyses conidia were germinated by shaking on a rotary shaker (New Brunswick) at 30 °C for 9–10 h. The germination media was usually Vogels minimal medium N, supplemented with either 20 mM potassium acetate or 2% sucrose. The original conidial inoculum was sufficient to give 0.1 mg dry wt conidia/ml of media. The germinated conidia were harvested by centrifugation ($3600 \times g$, 4 °C), washed with sterile distilled water (0 °C), and resuspended to 1.0 mg dry wt cells/mg in sterile distilled water.

Harvested cells were stored in crushed ice until used (usually within 3–4 h). A 9–10 h incubation of cells on 20 mM potassium acetate usually resulted in a 4-fold increase in dry weight as compared with a 16-fold increase for cells incubated on 2% sucrose.

Transport studies

Uptakes of ^{14}C-labelled substrates were undertaken essentially as previously described[30], but at 30 °C. The uptake mixture contained Vogels salts, cells (0.2 mg dry wt/ml), ^{14}C-labelled substrate (specific activity 2 Ci/mole), and sterile distilled water. The uptake of isotope was initiated by the addition of cells and aliquots (1 ml) were removed at 2-, 4-, 8- and 16-min intervals, filtered onto membrane filters, (Sartorius 0.45 μm), washed twice with equal volumes of ice cold distilled water, placed in scintillation vials with PCS (Nuclear Chicago Corp.) and dioxane (1:1, v/v) and counted in a scintillation spectrometer (Nuclear–Chicago Mark II). Under these conditions uptake remained linear for 8–16 min.

Extraction and chromatography

For the determination of the identity of transported substrate, cells were

incubated for 16 min (30 °C) in basic uptake mixtures. At the end of 16 min a 1-ml aliquot (0.2 mg dry wt cells) was removed, filtered, washed and counted as before. Simultaneously, the remaining cells (6.0 mg) were filtered, washed, resuspended in 5 ml distilled water and boiled for 20 min. The hot water filtrate was lyophilized and chromatographed by two-dimensional thin-layer chromatography[40]. Identity of labelled material was determined by radioautography in comparison to known standards.

Enzyme assays

To determine the levels of Krebs cycle enzymes, conidia were incubated in the same way as were conidia used in transport studies. After 9–10 h incubation in Vogel's minimal media N with either 20 mM potassium acetate or 2% sucrose, the germinated conidia were collected and washed by filtration. The washed cells were frozen until used. Thawed mycelial pads (in 0.1 M phosphate buffer (pH 7.0)) were later broken by repeated treatment in a tissue homogenizer (equipped with a Teflon plunger). The broken cell suspension was then centrifuged for 10 min at $3600 \times g$ (4 °C) and cell debris discarded. The supernatant was recentrifuged again (twice at $3600 \times g$ for 10 min) to remove as much whole cell debris as possible. The resulting cleared supernatant was then sonicated for 2 min (MSE sonicator) and centrifuged for 30 min at $48000 \times g$ to yield a particulate fraction (membranous material) and the soluble cytoplasmic material. The particulate fraction was resuspended in 0.1 M potassium phosphate buffer (pH 7.0). These fractions were then used for enzyme analysis.

Protein concentrations were determined by the method of Lowry *et al.*[31].

Citrate synthetase (EC 4.1.3.7) was measured by the method of Weitzman[32]; aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.1.41) by the method of O'Brien and Stern[33]; α-ketoglutarate dehydrogenase (EC 1.2.4.2) by the method of Kaufman[34]; succinate thiokinase (EC 6.2.1.4) by the method of Kaufman *et al.*[35]; succinate dehydrogenase (EC 1.3.99.1) by the method of King[36]; fumarase (EC 4.2.1.1) by the method of Racker[37]; malate dehydrogenase (EC 1.1.1.37) by the oxaloacetate-dependent reduction of NAD^+; malic enzymes (NADH and NADPH) (EC 1.1.1.38 and 1.1.1.40) by the malic acid-dependent reduction of NAD^+ and $NADP^+$; aspartase (EC 4.3.1.1) by the method of Racker[37]; glutamate–oxaloacetate transaminase (EC 2.6.1.1) by the α-ketoglutarate-dependent reduction of NAD^+ in the presence of aspartate; isocitrate lyase by the method of Kornberg[38]; and malate synthase by the method of Ornston and Ornston[39].

Chemicals

The dicarboxylic acid analogs were obtained from Sigma or Aldrich unless indicated otherwise. The inhibitors 2,4-dinitrophenol, dicyclohexylcarbodiimide (DCCD), NaN_3, NaF, iodoacetamide, sodium arsenite, *N*-ethylmaleimide, *p*-chloromercuribenzoate (PCMB), hydroxyquinoline-*N*-oxide (HOQNO) and amytal were obtained from Sigma. L-(+)-Tartarate and $K_3Fe(CN)_6$ were obtained from BDH, D-(+)-malate was obtained from Koch-Light and malonate was obtained from Eastman. DL-*erythro*-β-Hydroxyasparate, DL-*threo*-β-hydroxyasparatate, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and valinomycin were obtained from Calbiochem, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was

purchased from Pierce Chemical Co., and L-cysteic acid and L-homocysteic acid were purchased from Nutritional Biochemical Corp. Phenylsuccinate and *p*-nitrophenylsuccinate were the generous gifts of Dr S. I. Chavin (Duke University). [2,3-$^{14}C_2$]-Succinate, [2,3-$^{14}C_2$]fumarate, L-[U-^{14}C]malate, [1,4-$^{14}C_2$]tartarate, [2-^{14}C]acetate and [1,6-^{14}C]citrate were purchased from Amersham/Searle. Rutamycin was generously donated by Dr G. Rank (University of Saskatchewan).

RESULTS

Induction of dicarboxylate transport

Preliminary observations demonstrated that cells previously grown on sucrose as a sole carbon source were essentially devoid of the ability to transport dicarboxylic acids. However, when conidia were allowed to germinate on acetate minimal medium a striking increase in the ability to take up labelled succinate occurred after a 6-h incubation (Fig. 1). This increase always occurred after approximately a 3–4-fold increase in dry weight. These data indicated that this metabolite uptake system was strongly repressed in sucrose grown cells. Growth of cells in the presence of other carbon sources were not nearly as effective as acetate (Table I), furthermore, acetate expectedly induced rapid uptake of acetate, but not citrate. Succinate was not as effective as acetate, but it should be noted that conidia do not germinate as effectively on dicarboxylic acids as on acetate. *N. crassa* was found not to be able to use succinate, fumarate, L-malate or citrate as a sole carbon source. Acetate incubation induced succinate transport 50–100-fold over sucrose grown cells.

Nature of accumulating intermediates

90% of the radioactivity taken up from a typical labelled dicarboxylic acid intermediate was found to be soluble with little isotope being removed by oxidation

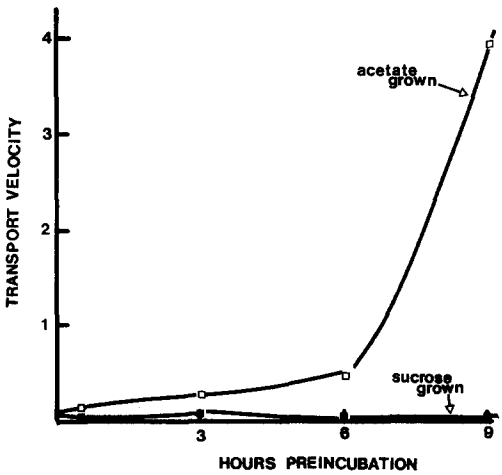

Fig. 1. [^{14}C]Succinate transport (nmoles/min per 2 mg dry wt cells, pH 5.8) by *N. crassa* conidia preincubated in Vogels minimal media N with 20 mM potassium acetate or 2% sucrose for the time periods indicated. Extracellular succinate at a 0.1 mM concentration.

TABLE I

TRANSPORT VELOCITIES OF CONIDIA OF *N. CRASSA* PREINCUBATED 9 h ON CARBON SOURCE INDICATED PRIOR TO [^{14}C]SUCCINATE, [^{14}C]ACETATE OR [^{14}C]CITRATE UPTAKE

Velocity values as nmoles/min per 0.2 mg dry wt cells with external concentration of labelled substrate at 0.1 mM.

Inducer (20 mM)	[^{14}C]*Succinate*	[^{14}C]*Acetate*	[^{14}C]*Citrate**
Acetate	0.14	3.53	0.0
Succinate	0.06	1.03	0.0
Citrate	0.02	0.60	0.0
Glycerol	0.08	0.62	0.0
Sucrose (2%)	0.003	—	0.0

* Since Vogels salts is a citrate phosphate buffer it was necessary to perform the citrate uptake under phosphate buffering conditions. No transport activity for citrate was observed.

(in each experiment less than 10% of the total radioactivity in the media was removed by the cell suspension). However, subsequent analysis of the hot water soluble conidial extract by thin-layer chromatography and radioautography[40], revealed only traces of tricarboxylic acid cycle intermediates. The majority (78–80%) of the intracellular label appeared as aspartate and glutamate while approx. 18% appeared as a heretofore unidentified neutral intermediate. These observations suggested that the increase in dicarboxylic acid transport observed following germination on acetate minimal media may be due to a secondary increase in tricarboxylic acid cycle activity, *i.e.* metabolic drag.

Levels of tricarboxylic acid cycle enzymes

To test the above hypothesis, conidia germinated on acetate or sucrose for

TABLE II

ASSAY OF KREBS CYCLE ENZYMES IN GERMINATED CONIDIA OF *N. CRASSA*
Conidia, were germinated by growing 9–10 h on the carbon source indicated prior to harvesting.

Enzyme	*Spec. act. (μmoles/min per mg protein)*		
	Sucrose grown	*Acetate grown*	*Acetate grown/ sucrose grown*
Citrate synthase	0.005	0.021	4.20
Isocitrate dehydrogenase	0.274	1.009	3.68
Aconitase	0.133	0.450	3.38
α-Ketoglutarate dehydrogenase	0.038	0.084	2.21
Succinate dehydrogenase	0.036	0.080	2.22
Fumarase	0.262	0.990	3.78
Malate dehydrogenase	7.000	16.000	2.29
Malic enzyme ($NADP^+$)	0.116	0.000	0.00
Glutamate-*O*-amino acid transaminase	0.313	0.175	0.56
Isocitrate lyase	0.138	2.145	15.50
Malate synthase	0.002	0.050	25.00

9–10 h were analyzed for tricarboxylic acid cycle enzymes. From Table II it may be seen that conditions which resulted in a 50–100-fold increase in succinate transport activity caused only a 3-fold increase in most of the tricarboxylic acid cycle enzymes and a 15–25 increase in the enzymes of the glyoxylate by-pass[41]. The "malic" enzyme

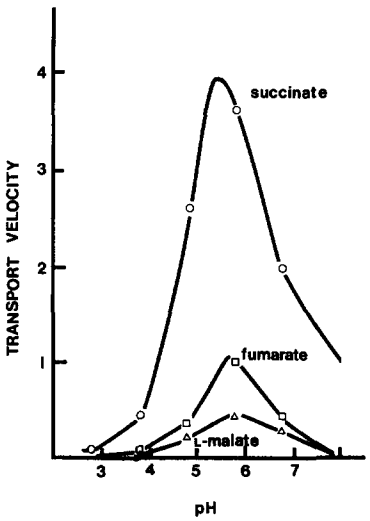

Fig. 2. Transport of succinate, fumarate, and L-malate (0.1 mM) by *N. crassa* as a function of pH. The conidia were germinated for 10 h on acetate minimal media as indicated in the text. Velocity indicated is nmoles/min per 2 mg dry wt cells.

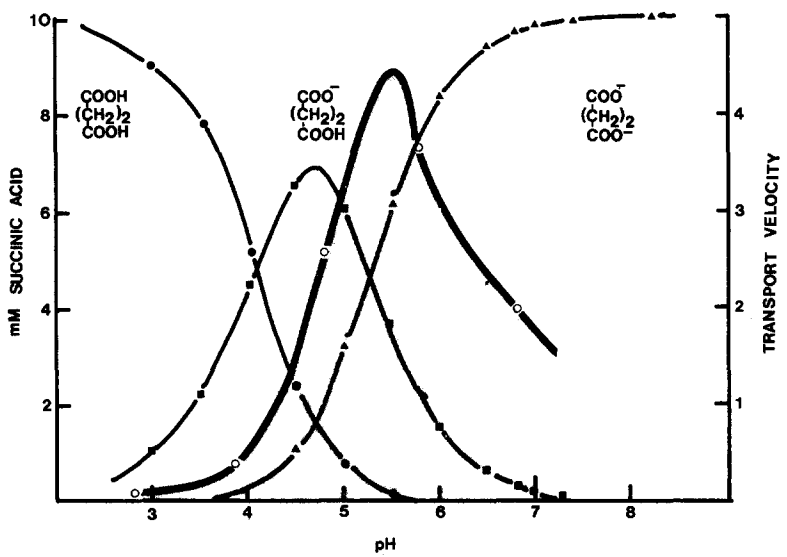

Fig. 3. Comparison of succinate transport (○—○) and distribution of ionic species of succinate as a function of pH. Transport velocity as in Fig. 2. ●—●, concentration of neutral species of succinate; ■—■, concentration of monoanionic species; ▲—▲, concentration of dianionic species.

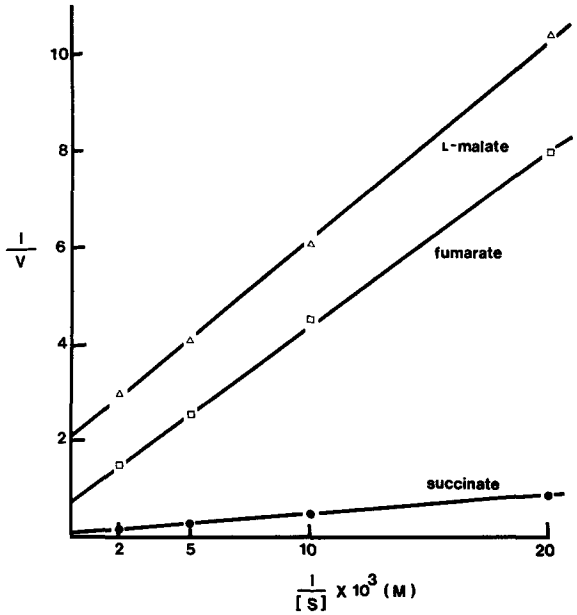

Fig. 4. Lineweaver–Burk plots of succinate, fumarate and L-malate transport (pH 5.8) by conidia germinated for 9–10 h in acetate minimal media. Velocity (v) values as nmoles/min per 0.2 mg dry wt cells.

was totally repressed as has been previously shown[42]. Therefore, these data suggested that growth on acetate caused the induction of a rate limiting step for metabolism of extracellular dicarboxylates—a dicarboxylic acid transport system.

Succinate transport as a function of pH

The succinate dehydrogenase of *N. crassa* has been previously shown to

TABLE III

KINETIC CONSTANTS FOR C_4-DICARBOXYLIC ACID TRANSPORT BY *N. CRASSA* CONIDIA (AT pH 5.8) PREINCUBATED 9–10 h ON 20 mM POTASSIUM ACETATE AS SOLE CARBON SOURCE

The constants given are averages of 3–10 separate determinations. V values as nmoles/min/mg dry wt cells. The K_i values indicated for each dicarboxylic acid were obtained by inhibition of transport of the C_4-dicarboxylic acid in parenthesis. The kinetic constants for L-aspartate are presumed to represent the summation of more than one transport system.

C_4-Acid	*K_m (mM)*	*V*	*K_i (mM)*
Succinate	0.34	13.0	0.12 (malate)
Fumarate	0.35	3.0	0.16 (malate)
L-Malate	0.16	1.7	0.12 (fumarate)
			0.16 (succinate)
L-Aspartate	0.46	16.6	0.22 (succinate)

exhibit two pH optima[44]. Succinate transport, however, exhibited only a single sharp pH optimum at approx. pH 5.5 (Fig. 2), as did both fumarate and L-malate. A similar pH optimum for these three dicarboxylic acids suggested that they share a single transport system. From a plot of both the rate of succinate transport and the ionic distribution of this substrate as a function of pH (Fig. 3) it can be seen that the substrate succinate is primarily available either as the singly or doubly ionized species.

Kinetics of dicarboxylic acid transport

The rates of succinate, fumarate and L-malate transport were examined as

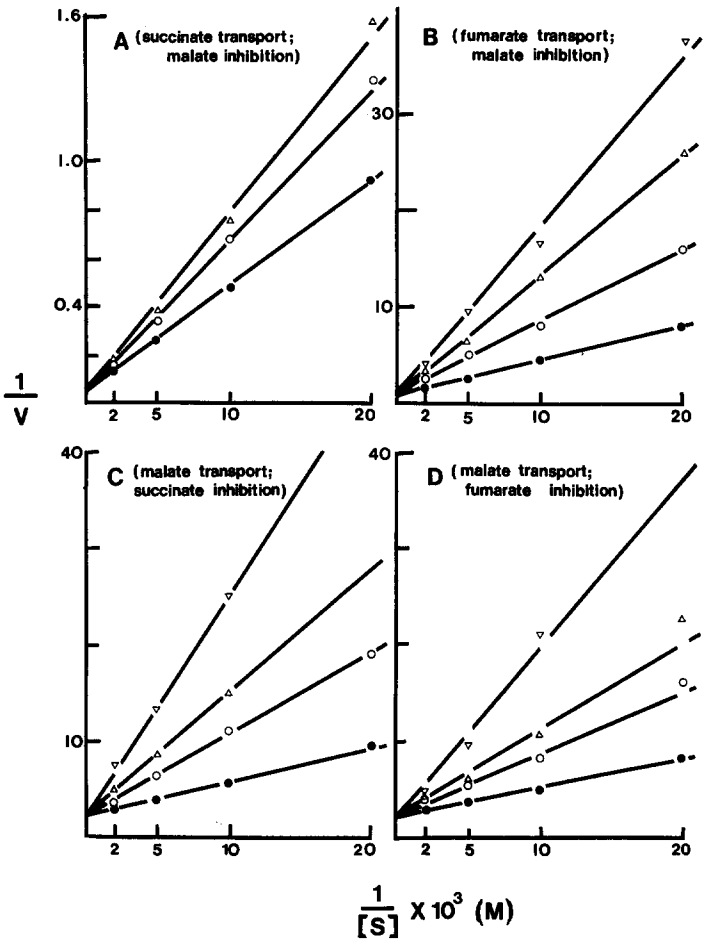

Fig. 5. Kinetics of inhibition of C_4-dicarboxylic acid transport (pH 5.8). A. [^{14}C]Succinate transport (●—●) with 0.2 mM (○—○) and 0.5 mM (△—△) L-malate. B. [^{14}C]Fumarate transport (●—●) with 0.2 mM (○—○), 0.5 mM (△—△) and 1.0 mM (▽—▽) L-malate. C. L-[^{14}C]Malate transport (●—●) with 0.2 mM (○—○), 0.5 mM (△—△) and 1.0 mM (▽—▽) succinate. D. L-[^{14}C]-Malate transport (●—●) with 0.2 mM (○—○), 0.5 mM (△—△) and 1.0 mM (▽—▽) fumarate. The velocity (v) values are nmoles/min per 0.2 mg dry wt cells. In each experiment the conidia had been germinated for 9–10 h on acetate minimal media.

a function of substrate concentration (0.05–0.5 μM) (Fig. 4). These three compounds were found to be transported by a system which exhibited typical saturation kinetics. Of the three dicarboxylic acids, succinate was transported with higher velocity and affinity than either fumarate or L-malate (Table III). These data suggested that this dicarboxylic acid transport system was preferentially reactive for succinate.

The three dicarboxylic acids competitively inhibited the uptake of one another (Fig. 5), confirming a single transport system with a broad substrate range. When each one of the three dicarboxylic acids were tested as a competitive inhibitor of the transport of the other two dicarboxylic acids (Table III), the K_i was found to be essentially the same as the K_m for transport. This again is evidence for a single transport system for these three carboxylic acids.

Effect of metabolic inhibitors

When acetate-germinated conidia of *N. crassa* were treated with various metabolic inhibitors and tested for succinate transport a differential response was observed (Table IV). Although transport could be effectively obliterated by proton

TABLE IV

INHIBITION OF SUCCINATE TRANSPORT (0.1 mM) BY VARIOUS METABOLIC INHIBITORS

Conidia which had been germinated for 10 h in acetate minimal media were preincubated for 30 min with the metabolic inhibitor prior to addition of labelled succinate. Velocity indicated is nmoles/min per mg dry wt cells at pH 5.8.

Metabolic inhibitor	*Concn (mM)*	*Transport velocity*	*% of control*
None	—	1.35	100.0
FCCP	1.0	0.00	0.0
CCCP	0.1	0.10	7.4
Dinitrophenol	1.0	0.00	0.0
NaN_3	1.0	0.10	7.4
HOQNO	1.0	1.35	100.0
K_3FeCN_6	1.0	1.90	141.0
K_3FeCN_6	10.0	1.25	92.5
DCCD	1.0	0.40	29.6
NaF	10.0	1.85	137.0
Gramicidin-D (or NF)*	0.1	1.20	96.0
Valinomycin	0.1 (mg/ml)	1.22	96.1
Rutamycin	0.1 (mg/ml)	1.27	94.0
Amytal**	10.0	0.50	30.3
Iodoacetamide	1.0	0.46	34.0
Iodoacetamide	10.0	0.00	0.0
Arsenite	10.0	1.05	77.7
PCMB	0.1	1.05	77.7
PCMB	1.0	0.15	11.0
N-Ethylmaleimide	0.1	1.05	77.7
N-Ethylmaleimide	1.0	0.12	9.0

* Gramicidin was dissolved in ethanol and the appropriate ethanol control was performed.

** Amytal was solubilized in dimethylsulfoxide and the appropriate dimethylsulfoxide control was performed.

conductors (FCCP, CCCP, 2,4-dinitrophenol, and NaN_3), it was not adversely effected to any great extent either by respiratory chain inhibitors ($K_3Fe(CN)_6$, amytal, HOQNO), ATPase inhibitors (NaF, DCCD), or ionophores (valinomycin, gramicidin D, rutamycin). Although gramicidin is known to conduct protons as well as K^+ and Na^+ in mammalian systems little effect was found in this system. Succinate transport was effectively inhibited by sulfhydryl reagents (iodoacetamide, PCMB, *N*-ethylmaleimide), suggesting that protein sulfhydryl groups were present in this transport system, whereas the lipoic acid reagent, arsenite was only mildly inhibitory.

Stereospecificity of dicarboxylic acid transport

The substrate requirements for dicarboxylate transport were examined with the use of a wide range of carboxylic acid analogues as potential competitive inhibitors. From the results in Table V several conclusions could be drawn. First, this transport system responds primarily to C_4-dicarboxylic acids. Carbon chain elongation (*e.g.* glutarate, glutaconate and iminodiacetate) resulted in poor competitive inhibition, whereas chain shortening (*e.g.* malonate) did not as drastically reduce the compounds ability to competitively inhibit. Citrate and *cis*-aconitate (C_6-tricarboxylic acids) were not competitive inhibitors. Second, carbons 2 or 3 of the C_4-carbon chain (but not both) could be effectively substituted (–Br, –OH, $-CH_3$, or

TABLE V

INHIBITION OF SUCCINATE TRANSPORT (pH 5.8) BY VARIOUS ANALOGS OF C_4-DICARBOXYLIC ACIDS

Transport of [^{14}C]succinate as described in Methods and Materials.

Analog (10 mM)	*% inhibition*	*Analog (10 mM)*	*% inhibition*
None	0	Succinamide	30
Citrate	0	*N*-Hydroxysuccinimide	35
cis-Aconitate	0	DL-*β*-Methylaspartate	39
meso-Tartarate	5	Mesaconate	46
N-Hydroxysuccinimide	5	Fumaronitrile	48
DL-Tartarate	8	Malonate (1 mM)	53
Phenylsuccinate	8	Mercaptosuccinate	55
Citramalate	9	Methylsuccinate	66
Maleate	9	*cis*-Oxaloacetate	67
Itaconate	10	Glutaconate	70
Dihydroxymaleate	14	L-Malate	76
DL-*threo*-*β*-Hydroxyaspartate	16	Succinamate	77
p-Nitrophenylsuccinate	18	*α*-Ketoglutarate	77
Citraconate	18	Monomethylsuccinate	78
Homocysteiate	18	D-Malate	84
D-Aspartate	19	Succinate	84
Iminodiacetate	20	Fumarate	84
Succinonitrile	22	Bromosuccinate	95
Dibromosuccinate	22	Succinyl chloride	95
Cysteiate	24		
Acetylene dicarboxylate	28		
L-Aspartate	30		

-SH) without a sizable loss in substrate recognition. DL-Tartarate was a very poor competitive inhibitor of succinate transport. In addition *N. crassa* was found to be unable to take up this metabolite from the media (unpublished data). Third, substitutions with large groups (*e.g.* phenyl) or substitutions at positions 2 and 3 were found to be unacceptable as inhibitors of succinate transport. Fourth, with the fumarate analogues, *trans*-isomers were requisite for recognition. Substitutions on the *cis*-isomers (*e.g.* dihydroxymaleate or citraconate) however, were found to slightly increase the inhibitory activity of otherwise inactive analogues. Also further carbon chain distortion (*e.g. cis*-oxaloacetate and acetylene dicarboxylate) lessened transport recognition. Fifth, the substitution of a single carboxyl group as either the acyl chloride, methyl ester or amide did not drastically reduce the activity as a competitive inhibitor. However, an alteration or substitution of both the carboxyl groups as either the diazide or diamide completely precluded its efficacy as a competitive inhibitor. Good inhibition by succinyl chloride seemingly indicated that large electro-negative groups at the terminus of the C_4-acid chain were desirable for the transport reaction to occur. L-Cysteic acid was also recognized to the same degree as L-aspartate suggesting that an acidic group (not necessarily a carboxyl) was required at the chain terminus.

The inhibition by malonate represents a somewhat special case deserving further comment. At low concentrations(1–5 mM) malonate was an effective competitive inhibitor (Fig. 6). At concentrations exceeding 5 mM however, the kinetics

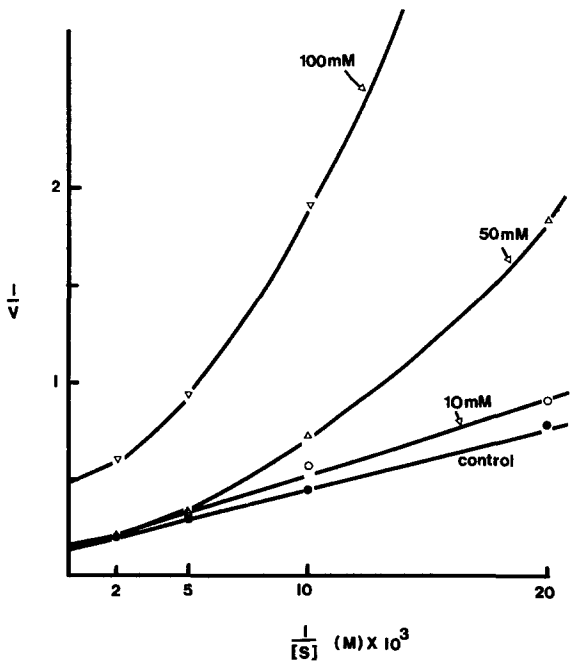

Fig. 6. Kinetics of malonate inhibition of succinate transport (pH 5.8) by *N. crassa* conidia germinated 10 h on acetate minimal media. Malonate concentrations are indicated in the figure. Velocity (v) values are nmoles/min per 0.2 mg dry wt cells.

became extremely complex. Although we do not interpret these kinetics mechanistically as yet, they do serve to illustrate that we are describing effects unrelated to further metabolism since it has been reported that the succinic dehydrogenase of this organism is only competitively inhibited by malonate[44].

DISCUSSION

N. crassa possesses a dicarboxylic acid transport system which is revealed by induction on acetate. As such it represents a genetically regulated system which is either catabolite repressed by sucrose or some close metabolite or is induced by acetate or one of its metabolites. Dicarboxylic acid transport is catabolite repressed in *E. coli*[9–11], *B. subtilis*[15,16], *A. vinelandii*[19], and *S. typhimurium* (Kay, W. W., unpublished data). Incubation in acetate-containing media has previously been shown to induce the glyoxylate cycle in *N. crassa*[41,42]. In *E. coli* this pathway has been shown to be derepressed by growth on acetate due to lowered levels of the corepressor phosphoenolpyruvate[45]. A glycolytic intermediate has also been implicated as a corepressor of the glyoxylate shunt in *N. crassa*[46]. In any case the products of this pathway are the dicarboxylic acids succinate and L-malate, which perhaps act as inducers for transport. Gratuitous induction of dicarboxylic acid transport by tartarate has been demonstrated in *E. coli*[9] and L-malate is felt to be the inducer for dicarboxylic acid transport in *B. subtilis*[15,16].

We have been unable to demonstrate any active accumulation of dicarboxylic acids in *N. crassa* due primarily to further metabolism. With the exception of the malate dehydrogenase (*ma*-1) mutant, tricarboxylic acid cycle mutants are presently unavailable and our attempts to isolate these have been repeatedly unfruitful. We have also found that malate dehydrogenase mutants metabolize rather than accumulate dicarboxylic acids added exogenously, however this is not surprising since at least two malate dehydrogenases have been reported in other eucaryotes[47] and in *N. crassa*[48]. Furthermore we have been unable to selectively inhibit metabolism with any of the analogues used in this study—although we have effectively inhibited dicarboxylate transport. Treatment of *N. crassa* conidia with a potent inhibitor of succinate dehydrogenase in *Ustilago maydis*[49,50] (carboxin) significantly reduced the rate of accumulation and metabolism of extracellular succinate, but did not result in the accumulation of label as succinate. As a result the question of active transport or facilitated diffusion as a transport mechanism for C_4-acids in *N. crassa* remains open.

Since metabolic inhibitors of proton conduction effectively obliterated succinate transport in these studies, it is tempting to conclude that active transport does in fact occur—perhaps linked to an electrogenic gradient, however, such a conclusion would be premature in light of possible secondary effects of such membrane permeants. Potent inhibition by iodoacetamide, PCMB and *N*-ethylmaleimide indicates the presence of a sulfhydryl group vital for dicarboxylate transport.

From the kinetics of competitive inhibiton, pH optima and malonate inhibition, we conclude that a single dicarboxylic acid transport system is operative in *N. crassa* for transport of the substrates succinate, fumarate, L-malate and L-aspartate. This order of recognition is somewhat similar to that reported for *E. coli*[8,9,11]. The inclusion of L-aspartate as a substrate of the C_4-dicarboxylic acid transport system is based on its ability to competitively inhibit transport of labelled

succinate. That succinate does not greatly inhibit L-aspartate transport under similar conditions (unpublished data) suggests that the totality of L-aspartate transport is due to more than one system. This is consistent with previous studies of L-aspartate transport in *Neurospora*[51]. Since the transport system described in this study likely transports only the negatively charged C_4-dicarboxylic acids, it is apparently different from the system which has been shown to transport the neutral[52] species of L-aspartate. Furthermore L-aspartate transport in *N. crassa* mycelia occurs by a system with different kinetic parameters, under cultural conditions which represses dicarboxylic acid transport, and is not competitively inhibited by tricarboxylic acid cycle dicarboxylic acids[53].

The elucidation of the number of transport systems operable here would be substantiated by the isolation of a dicarboxylic acid transport mutant. However, several attempts so far in this regard have been fruitless.

There are three molecular forms of the dicarboxylic acids (neutral, monoanionic and dianionic) each of which can predominate at some point within the pH growth range of *N. crassa*. It is of significance then that at the apparent optimum pH (5.5) for dicarboxylic acid transport succinate exists as a mixture of primarily the monoanionic (37%) and dianionic (61%) molecular forms[54], however, the pH curves span a broad area of anion concentration. Rapid uptake of succinate occurs only in the pH range at which it is ionized. This suggests that at least one ionized form of succinate (also L-malate and fumarate) is the molecular species transported. It should also be considered that pH effects can be manifold, the effect on both the transport system and membrane being of great importance. Contrary to the findings for L-aspartate transport[52], the pH sensitive component(s) of succinate transport could be manifest at a significantly higher pH than the pK value of membranes. Thus, the pH sensitive component(s) in succinate transport may be attributed to some other molecule(s) more directly involved in the transport process. It should be noted however, that this suggestion is speculative at this point as all pH effects could simply be due to substrate ionization. That substrate analogues with both carboxyl groups substituted are poor competitive inhibitors whereas analogues with singly substituted carboxyl groups are good competitive inhibitors of succinate transport agrees with the hypothesis that the substrate must be ionized for effective transport. We have previously come to a similar conclusion with succinate transport in *B. subtilis*[16].

The transport of carboxylic acids in *N. crassa* appears to be different from that described for mitochondria[26,27]. Citrate is not permeable to *N. crassa* and hence cannot serve as an exchange metabolite. Also it is unlikely that a dicarboxylic acid would exchange for another as a mechanism to drive inward flux since this would result in no net gain to the cell. We know that pyruvate carboxylase mutants require and take up tricarboxylic acid cycle dicarboxylic acids rapidly (unpublished data).

N. crassa is unable to use the tricarboxylic acid cycle intermediates as a sole carbon and energy source yet harbours a mechanism for their entry. This organism can grow normally on glutamate[46], which demonstrates that catabolism *via* the tricarboxylic acid cycle is not restricting growth on C_4-acids and therefore identifies the dicarboxylic acid transport system as rate-limiting for growth. The dicarboxylic acid transport system must then play a scavenger role in metabolism, either for exogenous acids in the environment and/or possibly as a recapture mechanism for

those dicarboxylic acids which have escaped or leaked from the cell interior.

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