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THE INDUCIBLE TRANSPORT OF DI- AND TRICARBOXYLIC ACID ANIONS ACROSS THE MEMBRANE OF *AZOTOBACTER VINELANDII*

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SUMMARY

1. Based on oxidation and accumulation studies of di- and tricarboxylic acid anions, the presence of at least four different inducible translocating systems in *Azotobacter vinelandii* is demonstrated.

2. Inducible transport is found for the following groups of anions: (i) succinate, fumarate, L-malate and oxaloacetate; (ii) citrate and isocitrate; (iii) 2-oxoglutarate; (iv) malonate. More than one translocator can be induced simultaneously. Oxidation of pyruvate, D- and L-lactate and acetate is constitutive.

3. Growth on Krebs-cycle intermediates is accompanied by an increase in the activity of 'malic' enzyme and oxaloacetate decarboxylase.

4. The inducible uptake of the different anions is energy dependent.

INTRODUCTION

Transport of Krebs-cycle intermediates across bacterial membranes has been studied by only a few investigators[1-8], in contrast to the large attention given to transport of sugars and amino acids[9,10].

The evidence for impermeability of bacterial membranes towards di- and tricarboxylic acid anions comes mostly from adaptation studies, in which oxidation of a substrate is a proof of permeability. However, an important step in the continuous operation of the Krebs cycle when di- or tricarboxylic acid anions are the substrates is a decarboxylation necessary to provide acetyl-CoA. Whether the necessary enzyme is present or absent in cells that do not oxidize Krebs-cycle intermediates has not usually been examined (see, however, ref. 11).

Another fact that has been overlooked in some studies[12,13] in which the actual uptake of the anions was measured is that in actively metabolizing cells most of the substrate added is converted to CO_2 and disappears subsequently from the system. However, WILLECKE AND PARDEE[8] reported citrate uptake in *Bacillus subtilis*, lacking aconitate hydratase.

In order to study systematically the properties of possible translocating systems available for Krebs-cycle intermediates, a study was undertaken with *Azotobacter*

Abbreviation: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

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vinelandii. Some 20 years ago KARLSSON AND BARKER[14] studied adaptation of Azotobacter cells to various carbon sources, while STONE AND WILSON[15] and REPASKE AND WILSON[16] measured the enzymic activity of extracts of adapted cells. This study was extended by REPASKE *et al.*[5], who showed that adaptation is sensitive to chloramphenicol and other inhibitors of protein synthesis. Furthermore, they investigated the specificity of the induction.

If one compares the few extensive reports dealing with the specificity of adaptation[1,4,5,7], no general picture emerges, compared for instance with the specificity of the di- and tricarboxylic acid anion translocators in mitochondria[17]. One of the aims of this study is to show that in Azotobacter different translocators with high specificity can be induced for di- and tricarboxylic acid anions. Furthermore, it will be shown that a decarboxylating enzyme is induced when cells are grown on Krebs-cycle intermediates.

Finally, the measurement of the uptake of Krebs-cycle intermediates is discussed and some difficulties connected with this problem are indicated.

METHODS

A. vinelandii (strain ATCC 478) was grown at 30° on a slightly modified Burk's nitrogen-free medium, containing 25 mM potassium phosphate, 0.6 mM $CaCl_2$, 0.6 mM Na_2SO_4, 3.5 mM NaCl, 0.8 mM $MgCl_2$, 0.03 mM Na_2MoO_4, 0.003 mM $CuSO_4$ and 0.24 mM $FeCl_3$, final pH 7.0, and containing 25 g/l of the carbon source used. Cells were grown for 24 h on a rotatory shaker in 3-l flasks (final volume 1 l) and harvested each day at an $A_{580\ nm}$ of 1.2–1.4 cm^{-1} (logarithmic phase). After washing twice with cold distilled water, cells were suspended in water at a protein concentration of 10–15 mg/ml.

Adaptation

Adaptation to various carbon sources was carried out essentially as described by REPASKE *et al.*[5]. Freshly prepared cells (approx. 15 mg protein) were incubated for 2 h on a rotatory shaker at 30° in a medium containing 25 mM potassium phosphate, 1 mM $MgCl_2$, 60 mg glucose and the inducer, in a final volume of 100 ml, and final pH of 7.0. After centrifugation and washing cells were resuspended in 3 ml of distilled water.

Oxygen uptake

Oxygen uptake was monitored with a Clark-type electrode (Gilson Medical Electronics Oxygraph). All reactions were carried out in 25 mM Tris–HCl buffer (pH 7.6) at 25°. The reaction was started by adding the substrate (potassium salt) and all rates are corrected for endogenous respiration (usually 0.05 μatom O/min per mg protein).

Ion accumulation

Accumulation of ^{14}C-labelled substrates was measured by the centrifugation–filtration technique of WERKHEISER AND BARTLEY[18] as modified by HARRIS AND VAN DAM[19]. Bacteria were incubated at a concentration of approximately 1–2 mg protein/ml in a medium containing ^{14}C-labelled substrate and ^3H_2O. 0.3-ml samples

were transferred to small centrifuge tubes that contained 50 μl silicone oil (2 parts Wacker Chemie AR 100, density 1.05 g/ml, 1 part Dow Corning 200, density 0.99 g/ml) layered on top of 30 μl 1.5 M $HClO_4$. Each sample was centrifuged immediately in a microcentrifuge (Coleman Model 6-811). ^{14}C and ^{3}H radioactivities were determined by dissolving 25-μl samples in 10 ml fluid consisting of toluene–ethanol (3:1, v/v) with 2 g 2,5-diphenyloxazole *plus* 25 mg 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per l.

Internal substrate concentrations can be calculated from the formula:

$$f = [^{14}C/^{3}H]_{acid} : [^{14}C/^{3}H]_{supernatant}$$

where f is the factor by which bacteria concentrate the substrate, with a correction for the adhering water (see RESULTS).

Enzyme assays

Freshly harvested cells (150 mg protein in 10 ml 25 mM Tris–HCl buffer, pH 7.6) were sonicated for 90 sec on a Branson sonifier. After centrifugation at 20000 $\times$ g for 30 min, the supernatant was centrifuged 60 min at 100000 $\times$ g. The resulting supernatant contained most of the Krebs-cycle enzymes. The pellet contained succinate oxidase, L-malate oxidase and NADH oxidase activity.

Citrate synthase (EC 4.1.3.7) was measured as described by SRERE[20] by following the increase in absorbance at 412 nm using 5,5'-dithio-bis-(2-nitrobenzoic acid). Aconitate hydratase (EC 4.2.1.3) and fumarate hydratase (EC 4.2.1.2) were measured according to the method of RACKER[21]. 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) and pyruvate dehydrogenase (EC 1.2.4.1) were determined by measuring the increase in absorbance of NADH at 340 nm, as described by REED AND MUKHERJEE[22]. Isocitrate dehydrogenase (NADP) (EC 1.1.1.42) was determined according to the method of CLELAND *et al.*[23]. Malate dehydrogenase (decarboxylating) (NADP) (EC 1.1.1.40) was determined as described by HSU AND LARDY[24]. Succinate dehydrogenase (EC 1.3.99.1) and L-malate oxidase (EC 1.1.3.3) were determined polarographically in the particulate fraction (that contains the respiratory chain) in 25 mM Tris–HCl buffer (pH 7.6). Oxaloacetate decarboxylase (EC 4.1.1.3) was measured by following the decrease of oxaloacetate absorbance at 290 nm or by following the decrease in absorbance at 340 nm in a medium containing 25 mM Tris–HCl buffer (pH 7.6), 2.4 mM oxaloacetate, 1.2 mM $MgCl_2$, 0.1 mM NADH and lactate dehydrogenase (1.8 enzyme units).

Specific activities are expressed in units of μmoles substrate metabolized/min per mg protein at 25°.

Assay of carboxylic acid anions

To determine the amount of metabolites present in Azotobacter cells, the reaction mixture was fixed by adding $HClO_4$, final concentration 5 %. After removal of the precipitated protein, a sample of the supernatant was neutralized with KOH–Tris to pH 7.6. After freezing and thawing, $KClO_4$ was removed by centrifugation. In the neutralized extracts, metabolites were measured spectrophotometrically, using an Aminco-Chance double-beam spectrophotometer.

Citrate, L-(+)-isocitrate, 2-oxoglutarate, oxaloacetate and pyruvate were deter-

mined as described in refs. 25–29, respectively. Fumarate and L-malate were determined according to the method of SCHONER[30], omitting the last step.

Assay of CO_2

Reactions were carried out in Warburg vessels. The reaction mixture in the main vessel contained 25 mM Tris–HCl buffer, 2 mM ^{14}C-labelled substrate and 0.5 mg protein in a final volume of 1 ml and final pH 7.6. The reaction was started by the addition of cells and stopped by the addition of 0.10 ml 2.5 M H_2SO_4 from the side-arm. The centre well contained 0.01 ml 1 M hyamine hydroxide in methanol on a strip of Whatman No. 1 paper edged with paraffin wax[31]. After shaking for 1 h the filter paper was counted in a liquid scintillation counter as described earlier.

Determination of protein

Protein was determined by the biuret method as described by GORNALL *et al.*[32].

Special chemicals and enzymes

All purified enzymes were obtained from Boehringer und Soehne. [1,4-$^{14}C_2$]-Succinate, [2,3-$^{14}C_2$]succinate, L-[U-^{14}C]malate and ^3H_2O were obtained from the Radiochemical Centre, Amersham. [1,5-$^{14}C_2$]Citrate and [8-^{14}C]adenosine 5'-diphosphate were obtained from Cal. Atomic. [*carboxyl*-^{14}C]Dextran and [*carboxyl*-^{14}C]inulin were obtained from New England Nuclear. Compounds used as substrates or inducers were obtained from the following sources: acetate, malonate, succinate and citrate, British Drug Houses; 2-oxoglutarate, fumarate, pyruvate and oxaloacetate, Boehringer und Soehne; DL-isocitrate, D-lactate and L-lactate, Sigma Chemical Co.; L-malate, Koch-Light Laboratories; malonic acid, Fluka. 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was a gift from Dr. R. H. Büchel.

RESULTS

Adaptation

Table I shows the oxidation velocity of different carboxylic acid anions by *A. vinelandii*, grown on various carbon sources. The rates are the steady-state rates of oxygen consumption reached after about 1 min. In Table II is shown systematically the oxidation pattern of the different Krebs-cycle intermediates and related carboxylic acid anions in relation to the carbon source to which the cells were adapted. It appears that one group of substrates is always oxidized, another group only after adaptation to the specific substrate. A differentiation can be made between oxidative systems for C_3-, C_4- and C_5-dicarboxylic acid anions and C_6-tricarboxylic acid anions. Monocarboxylic acid anions are oxidized without adaptation. A clear analogy exists with the mitochondrial system, except for the case of malonate.

The results are different from those reported by several authors[1,4,5,7], in which overlap between the groups mentioned above occurs (in other organisms). One reason can be contamination of the substrates used. For instance, DL-isocitrate contains 2–4 % succinate, while sodium malonate (British Drug Houses) contains about 0.5 % acetate. Because of the low K_m values for oxidation (see below) these contaminations may play an important role when the rate of oxygen uptake is measured polarographically.

TABLE I

OXIDATION OF CARBOXYLIC ACID ANIONS BY *Azotobacter vinelandii* GROWN ON VARIOUS CARBON SOURCES

Oxidation was measured as described in METHODS. The velocity is the steady-state velocity after about 1 min, expressed in μatoms O/min per mg protein. Substrates were added at a concentration of 6 mM, except for pyruvate, lactate, citrate and isocitrate, when 12 mM was used.

Substrate	*Carbon source during growth*		
	Sucrose	*Succinate*	*Citrate*
Succinate, fumarate, L-malate, oxaloacetate	0.1	1.8	0.1
Citrate, isocitrate	0.1	0.05	1.50
2-Oxoglutarate	0.05	0.05	0.05
Pyruvate, D- or L-lactate	1.70	1.40	1.00
Malonate	0.05	0.05	0.05
Acetate	1.50	1.60	1.30

TABLE II

RELATIONSHIP BETWEEN OXIDATION OF CARBOXYLIC ACID ANIONS AND CARBON SOURCE

Adaptation and oxidation were measured as described in METHODS. +, oxidation; −, no oxidation.

Substrate	*Carbon source*					
	Succinate, fumarate, L-malate, oxaloacetate	*Malonate*	*2-Oxo-glutarate*	*Citrate, isocitrate*	*Pyruvate, lactate*	*Acetate*
Succinate, fumarate, L-malate, oxaloacetate	+	−	−	−	−	−
Malonate	−	+	−	−	−	−
2-Oxoglutarate	−	−	+	−	−	−
Citrate, isocitrate	−	−	−	+	−	−
Pyruvate, lactate	+	+	+	+	+	+
Acetate	+	+	+	+	+	+

Enzyme activities

As mentioned in INTRODUCTION, when using non-oxidation as a proof of impermeability, one must check that all the enzymes necessary for oxidation are present. In Table III the activity is shown of Krebs-cycle and related enzymes in extracts of Azotobacter grown on different carbon sources. Only 'malic' enzyme and oxaloacetate decarboxylase differ significantly in activity in different cells. 'Malic' enzyme varies between 0.03 and 0.06 μmole/min per mg protein in sucrose-grown cells and between 0.20 and 0.30 μmole/min per mg protein in succinate-grown cells. Cells that use di- or tricarboxylic acid anions (except perhaps malonate) as a carbon source have a higher level of a decarboxylating enzyme. It is questionable, however, if oxaloacetate decarboxylase is active *in vivo* because it only shows activity in Tris–HCl buffer but not in phosphate buffer.

It remains difficult to decide from these results alone if the difference in activity

TABLE III

ACTIVITY OF KREBS-CYCLE AND RELATED ENZYMES IN *Azotobacter vinelandii* GROWN ON DIFFERENT CARBON SOURCES

Activity of all the enzymes was measured as described in METHODS. Specific activity is expressed in μmoles/min per mg protein at 25°.

Enzyme	*Carbon source*			
	Sucrose	*Pyruvate*	*Succinate*	*Citrate*
Citrate synthase	0.51	0.59	0.59	0.53
Aconitate hydratase	1.43	2.26	1.85	1.06
Isocitrate dehydrogenase	1.30	1.70	1.0	1.65
2-Oxoglutarate dehydrogenase	0.18	0.12	0.36	0.20
Succinate dehydrogenase	0.42	0.26	0.26	0.76
Fumarate hydratase	4.90	4.80	3.30	5.10
Malate dehydrogenase	5.10	5.0	4.5	5.0
'Malic' enzyme	0.04	0.06	0.20	0.15
Oxaloacetate decarboxylase	0.60	0.60	4.9	5.5
Pyruvate dehydrogenase	0.10	0.09	0.14	0.10

TABLE IV

OXIDATION OF CARBOXYLIC ACID ANIONS BY *Azotobacter vinelandii*

Oxidation is measured as described in METHODS. Cells were adapted simultaneously to all compounds indicated. Succinate, 0.5 mM; 2-oxoglutarate, 1 mM; magnesium citrate, 2 mM; malonate, 10 mM. Oxidation velocity is expressed in μatoms O/min per mg protein.

Adapted to	*Substrate*				
	Succinate	*2-Oxo-glutarate*	*Citrate*	*Malonate*	*Pyruvate*
—	0.05	0.05	0.05	0.10	1.50
Succinate	2.00	0.05	0.05	0.10	1.60
2-Oxoglutarate	0.05	1.30	0.05	0.05	1.40
Citrate	0.03	0.10	1.75	0.05	1.50
Malonate	0.05	0.05	0.05	1.30	1.55
Succinate + 2-oxoglutarate	1.50	1.15	0.05	0.05	1.50
Succinate + malonate	1.00	0.15	0.20	1.15	1.50
2-Oxoglutarate + citrate	0.15	1.25	1.40	0.10	1.50
Succinate + 2-oxoglutarate + citrate	1.00	1.15	1.20	0.05	1.50

of 'malic' enzyme can explain the difference in behaviour between succinate and sucrose-grown cells, *e.g.* during succinate oxidation.

Specificity of the translocating systems

Although there seems to be a correlation between oxidation of di- and tricarboxylic acid anions and the synthesis of 'malic' enzyme or oxaloacetate decarboxylase, it is clear from Tables II and III that specific translocating systems must be synthesized during adaptation. It would be difficult to explain otherwise why succinate-grown cells, possessing 'malic' enzyme, still cannot oxidize citrate or 2-oxoglutarate and *vice versa*.

The induction is sensitive to chloramphenicol (1 mg/ml) and rifampicin (20

μg/ml). Furthermore, preliminary experiments indicate that induction is quite specific. A C_4-dicarboxylic acid anion translocator is induced only by comparable concentrations of mesotartrate and D-malate, apart from the four anions shown in Tables I and II. Glutamate cannot substitute for 2-oxoglutarate, aspartate for oxaloacetate nor tricarballylate for citrate, at least in concentrations less than 5 times that of the specific anion.

As shown in Table IV, it is possible to induce each of the possible translocators alone or in combination. If succinate-grown cells (which oxidize succinate rapidly) are adapted to 2-oxoglutarate or citrate the rate of succinate oxidation remains constant. Thus one can make a cell that can oxidize all the carboxylic acid anions simultaneously.

A remarkable observation is the fact that the apparent K_m values for the substrate oxidation fall in groups that, as shown in Table V, contain the same anions as the groups inducing the different translocators. At the moment it is impossible to say what these K_m values stand for, because the whole process is a combination of transport and ten or more metabolic steps.

TABLE V

K_m VALUES FOR OXIDATION OF KREBS-CYCLE INTERMEDIATES BY VARIOUS AZOTOBACTER CELLS

Oxidation was measured as described in METHODS. K_m values were calculated from the steady-state velocity after approx. 1 min.

Substrate	*Induction by*	K_m (μM)
Succinate, fumarate, L-malate, oxaloacetate	Succinate, mesotartrate	100–200
2-Oxoglutarate	2-Oxoglutarate (with or without succinate)	150
Citrate, isocitrate	Citrate (with or without succinate)	2000
Pyruvate, lactate	Sucrose, succinate	3000
Acetate	Sucrose, succinate, citrate	30–60

Formation of CO_2 and Krebs-cycle intermediates

Fig. 1 shows the oxygen uptake and $^{14}CO_2$ formation when succinate-grown cells are oxidizing L-[^{14}C]malate. After a lag time of about 1 min, a steady-state consumption of oxygen and formation of CO_2 is found. The lag time is interpreted as the time required to build up a steady-state concentration of Krebs-cycle intermediates. Fig. 2 shows the result of the measurement of the intermediates present at different times after adding succinate to succinate-grown cells. Although the Krebs-cycle intermediates are measured in the total extract (outside *plus* inside) centrifugation experiments show that almost none of the intermediates is outside the cell.

A gradual building-up of various Krebs-cycle intermediates is seen. From the amount of oxygen disappearing at 18° (about 1250 natoms O/mg protein in 2 min) and CO_2 formed at 18° (about 750 nmoles CO_2/mg protein in 2 min), calculated from Fig. 1, one can conclude that more than 85 % of the malate disappearing is converted to CO_2 and H_2O by the reaction 1 malate + $6O \rightarrow 4CO_2 + 3H_2O$ in the first 2 min. The rest can be accounted for by the accumulation of intermediates.

Ion accumulation

In Fig. 3 is shown the uptake of succinate and citrate by sucrose- and succinate-grown cells. Although the uptake is given as a concentration factor of the substrate added, in reality it will be the sum of all the anions present as is also clear from Fig. 2. From this concentration factor one can calculate the inside concentration of all Krebs-cycle intermediates together after correcting for adhering water, measured in a way similar to the determination of the sucrose space in mitochondria. Intracellular water is calculated to be 5–6 μl/mg protein (see below).

It is seen from Fig. 3 that sucrose-grown cells are unable to concentrate added succinate or citrate. Succinate-grown cells in contrast can build up a concentration

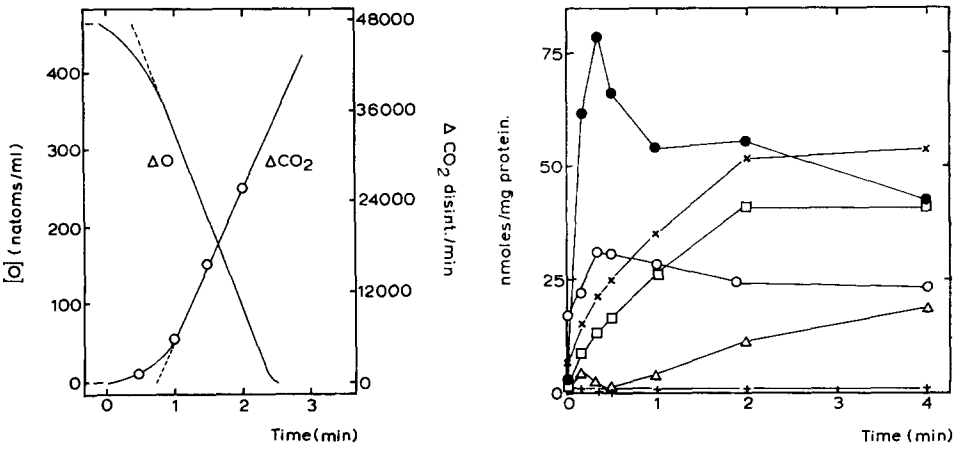

Fig. 1. Oxygen consumption and CO_2 formation during L-malate oxidation by succinate-grown cells. The Warburg vessel used for determination of CO_2 production contained 25 mM Tris–HCl buffer (pH 7.6), 2 mM L-[^{14}C]malate (specific activity 270 disint./min per nmole) and 0.47 mg protein/ml. Oxygen uptake of a suspension of cells containing 0.3 mg protein/ml was measured polarographically. Temp., 18°.

Fig. 2. Distribution of Krebs-cycle intermediates in time during oxidation of succinate by succinate-grown cells. The vessel contained 25 mM Tris–HCl buffer, 30 mM succinate and 1.3 mg protein/ml; final pH, 7.6. Temp., 25°. ●—●, fumarate; □—□, malate; △—△, oxaloacetate; ○—○, citrate; +—+, isocitrate or 2-oxoglutarate; ×—×, pyruvate.

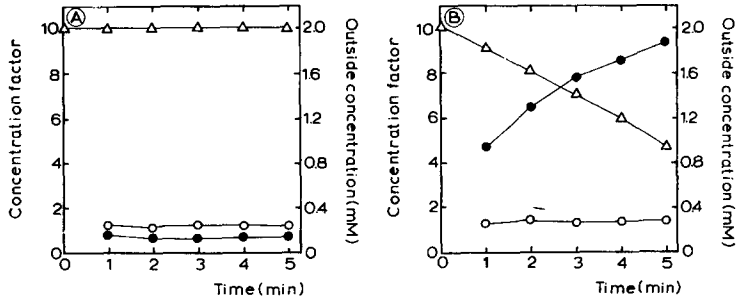

Fig. 3. Uptake of succinate and citrate by sucrose- and succinate-grown cells. Incubation medium contained 25 mM Tris–HCl buffer (pH 7.6), 1.5 mg protein/ml, and 2 mM [^{14}C]succinate or 2 mM [^{14}C]citrate. Temp., 18°. Air was bubbled through to keep the solution aerobic. A. Sucrose-grown cells. B. Succinate-grown cells; △—△, succinate concentration in medium; ○—○, concentration factor with 2 mM potassium citrate; ●—●, concentration factor with 2 mM potassium succinate.

gradient of 5–100, depending on the outside concentration. One has to be careful, however, in interpreting these data. From the data on oxygen uptake in the experiment of Fig. 3 (not shown here) one can calculate that approx. 1100 natoms O/min per mg protein have disappeared at 18°. This corresponds to about 160 nmoles substrate/min per mg protein for complete oxidation. Approximately the same value, 140 nmoles/min per mg protein, is obtained when the actual decrease is measured in the supernatant (see Fig. 3). However, according to Fig. 3 only 20 nmoles substrate/min per mg protein are taken up between 1 and 3 min, *i.e.* only 15 % of the added label is found inside the cell, while 85 % has disappeared as CO_2. It is clear that the real uptake velocity is greatly underestimated when metabolism is occurring.

In contrast to the findings of KAY AND KORNBERG[7], the accumulated anions are in the acid-soluble 'pool'. This is also borne out by the fact that our accumulation is insensitive to chloramphenicol.

The accumulation of succinate in succinate-grown cells is inhibited by fumarate, L-malate or oxaloacetate but not by 2-oxoglutarate or citrate. However, in cells

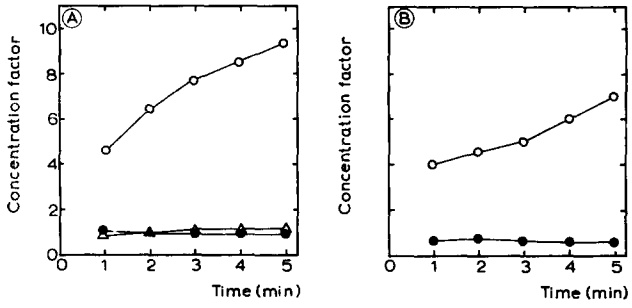

Fig. 4. Uptake of succinate and L-malate by succinate-grown cells under various energetic conditions. A. Cells were incubated in a medium containing 25 mM Tris–HCl buffer (pH 7.6), 1.5 mg protein/ml, and 2 mM [^{14}C]succinate. ○—○, aerobic, 18°; ●—●, anaerobic, 18°; △—△, aerobic, 2°. B. Cells, preincubated for 5 min with or without 100 μM TTFB, were incubated at 18° in a medium containing 25 mM Tris–HCl buffer (pH 7.6), with or without 100 μM TTFB, and 2 mM L-[^{14}C]malate. ○—○, malate; ●—●, malate + TTFB. 0.3-ml samples were withdrawn and centrifuged as described under METHODS.

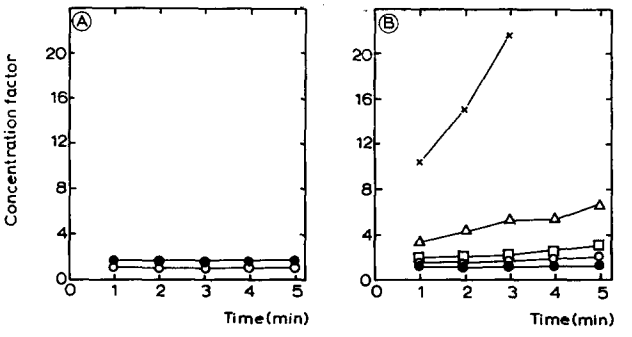

Fig. 5. Uptake of succinate and citrate by Azotobacter in the presence of pyruvate. The reaction was started by adding [^{14}C]succinate or [^{14}C]citrate to a medium at 18° containing 25 mM Tris–HCl buffer (pH 7.6), 10 mM pyruvate and protein. Air was bubbled through to keep the solution aerobic. A. Sucrose-grown cells, 3 mg protein/ml; ○—○, 0.2 or 2 mM succinate; ●—●, 0.5 mM citrate. B. Succinate-grown cells, 2 mg protein/ml; ●—●, 0.5 mM citrate; ○—○, 2 mM succinate; □—□, 1 mM succinate; △—△, 0.5 mM succinate; ×—×, 0.2 mM succinate.

adapted to succinate and citrate at the same time the accumulation of succinate is decreased by citrate but still not by 2-oxoglutarate. These results form the subject of a separate paper.

Intracellular water was determined in Azotobacter as the difference between extra-cellular water and total water. Total water was determined both by weighing and by equilibration with ^3H_2O. A value of 8.15 μl/mg protein was found by both methods. Determination of extra-cellular water proved to be more difficult. [^{14}C]-Dextran, [^{14}C]inulin, [^{14}C]ADP and [^{14}C]citrate, all thought to be impermeable, gave a concentration factor $\geqslant$ 0.8, just as GINZBURG[33] found for [^{14}C]inulin, [^{14}C]-sucrose and [^{131}I]polyvinylpyrrolidone. However, determination with serum albumin (measured by protein determination) indicated that 75–80 % of the total water is intracellular, giving a value of 5–6 μl/mg protein. Since the latter value agrees with other reports, it has been used throughout our study.

Energy dependence

A reason why succinate or other Krebs-cycle intermediates are not taken up by sucrose-grown cells could possibly be the lack of energy, if the translocator were energy dependent, as is the case in many bacterial systems.

We have measured the uptake of succinate at 2°, and under anaerobic conditions. In neither case do succinate-grown cells accumulate carboxylic acid anions, as is shown in Fig. 4. Also inhibitors of the succinate oxidation prevent accumulation. For instance, arsenite and fluoroacetate inhibit both succinate oxidation and succinate uptake by 95–100 %. Fig. 4 shows too that the uncoupler TTFB inhibits both uptake and oxidation. It can be shown that endogenous ATP synthesis in intact Azotobacter cells is inhibited 100 % by about 10 nmoles TTFB/mg protein (J. M. BAAK, unpublished results), when measured as described by HEMPFLING[34].

Because all the evidence points to a very close relationship between transport and metabolism (see also ref. 7), we designed the experiment shown in Fig. 5. Both sucrose- and succinate-grown cells can oxidize pyruvate and in this way supply energy, but only the succinate-grown cells accumulate succinate.

DISCUSSION

Transport of Krebs-cycle intermediates has been relatively little investigated in bacteria, even after the discovery of specific translocators for these anions in mitochondria (for review see refs. 17 and 35). One of the difficulties, reflected in most studies, is the fact that it is difficult to separate transport and metabolism of a specific carboxylic acid anion. Recently, however, WILLECKE AND PARDEE[8] reported inducible transport of citrate in a mutant of *B. subtilis*, lacking aconitate hydratase, while KAY AND KORNBERG[7] used mutants of *Escherichia coli*, deficient in some of the Krebs-cycle enzymes.

Many authors have speculated about the nature and mechanism of carrier systems (see refs. 10, 36 and 37). In mitochondria it has been firmly established that carboxylic acid anions are transported through the membrane *via* an exchange-diffusion system[17]. No definite conclusions have been drawn concerning bacteria. MITCHELL[38,39], however, suggested that the Krebs-cycle enzymes may play a role in transport through the bacterial plasma membrane.

In spite of the above-mentioned problems, several authors have postulated translocating systems for Krebs-cycle intermediates in bacteria. For instance, KAY AND KORNBERG[7] studied the C_4-dicarboxylic acid anion carrier in *E. coli*, WILLECKE AND PARDEE[8] the citrate carrier in *B. subtilis*, DAVIS[40] and VILLARREAL-MOGUEL AND RUIZ-HERRERA[12] a citrate carrier in *Aerobacter aerogenes*, LONDON AND MEYER[6] a malate carrier in Streptococcus and REPASKE *et al.*[5] possible carriers in *A. vinelandii*.

In this study we report the existence of at least four different translocators for di- and tricarboxylic acid anions in *A. vinelandii*.

The first translocator can transport succinate, fumarate, L-malate and oxaloacetate with approximately the same velocity. Furthermore, the uptake of one anion is inhibited by any of the three others, but not by other di- or tricarboxylic acid anions unless the respective translocator is induced. This translocator can be induced by any one of the four C_4-dicarboxylic acid anions mentioned as well as mesotartrate and D-malate. However, neither mesotartrate nor D-malate, nor other analogues and derivatives of C_4-dicarboxylic acid anions can inhibit the oxidation of the four C_4-dicarboxylic acid anions.

A second translocator exists for 2-oxoglutarate, inducible by this anion, but not by glutamate.

A third translocator transports citrate and isocitrate. It is induced by these anions but not by tricarballylate.

The fourth translocator can transport malonate only. However, this conclusion must be drawn with reserve because we have not investigated if perhaps simultaneously an enzyme is induced, necessary for the breakdown of malonate. In any case, succinate oxidation in succinate-grown cells is not inhibited by malonate.

Pyruvate, L-lactate, D-lactate and acetate are always oxidized and, therefore, transported by *Azotobacter vinelandii*, independent of the carbon source used for growth. Whether, for instance, the pyruvate transport is catalysed by a translocator is still a matter of debate[41], just as in the case of mitochondria[42].

The translocators can be induced in Azotobacter by adapting them to one of the carbon sources. As shown in Table IV, it is possible to induce the different translocators simultaneously, but in general the activity is somewhat less, compared with induction of one translocator alone. This may be due to lack of energy or to limitation of space for insertion of translocators, as suggested by HENNAUT *et al.*[43]. From our preliminary experiments we conclude that the inducer must be in contact with the outside of the cell to be active. Otherwise it is difficult to understand why sucrose- or pyruvate-grown cells do not contain the translocators, because measurements show that these cells contain appreciable amounts of Krebs-cycle intermediates during metabolism of sucrose or pyruvate. In fact, for induction of a C_4-dicarboxylic acid anion carrier only 0.1 mM added succinate is sufficient. It should be noted that in the adaptation experiments, glucose was added as a source of energy (up to 100 mM during adaptation to succinate) so that catabolite repression can not be the reason for the absence of the translocator during growth on sucrose. Experiments concerning these induction phenomena will be the subject of a forthcoming paper.

The translocators are not the only proteins synthesized by the cell during adaptation. Determination of the specific activities of all the Krebs-cycle and related enzymes suggests that 'malic' enzyme and also oxaloacetate decarboxylase are more active in the induced cells. Although the oxidation velocity of, for example, succinate

is increased by at least a factor of 15, comparing sucrose- to succinate-grown cells, the 'malic' enzyme activity increases by a factor of 5 or less. Perhaps the amount of a decarboxylating enzyme already present is enhanced for optimal functioning of the Krebs cycle. The formation of 'malic' enzyme is probably regulated at a site different from that responsible for the formation of the translocators, because 'malic' enzyme is induced both when the cells are grown on succinate and on citrate. A similar conclusion was reached by LONDON AND MEYER[6].

Now that the existence of translocators in Azotobacter for di- and tricarboxylic acid anions is firmly established, it is worthwhile to consider the resemblances and differences between bacterial and mitochondrial transport systems for these compounds.

In mitochondria it has been established that exchange-diffusion translocators exist with high specificity. In Azotobacter cells this is highly unlikely because substrates are oxidized nearly completely. In fact, only exchange of the substrate for bicarbonate seems to be possible. This seems reasonable because mitochondria are surrounded by the cytoplasm, whereas bacteria are surrounded by the outer world. Everything transported outwards is in principle 'lost'. At least, during oxidation the outside becomes acid with the same kinetics as the oxygen uptake (P. W. POSTMA, unpublished experiments).

Another indication that exchange diffusion cannot occur is the observation that succinate oxidation is inhibited 95–100 % by fluoroacetate and arsenite. This would not be the case if succinate–malate exchange could take place.

The close connection between transport and metabolism is another difference between mitochondrial and bacterial systems, also observed for instance by KAY AND KORNBERG[7] and WILLECKE AND PARDEE[8]. While in mitochondria no energy is needed for transport (at least no metabolic energy), Azotobacter can transport carboxylic acid anions only when energy is provided. Under anaerobiosis, at 0° or in the presence of uncoupler no transport is observed.

Transport in Azotobacter resembles mitochondrial transport in its specificity. However, one of the criteria for specific translocators[17], namely the existence of specific inhibitors, has not yet been met in bacteria. In fact none of the close analogues (like bromosuccinate, hydroxyfumarate, thiomalate, methylsuccinate, butylmalonate or mesotartrate) has any effect on the oxidation of Krebs-cycle intermediates.

However, it is clear from our results that different carrier systems must exist and are induced during adaptation in a way sensitive to chloramphenicol. The idea of Mitchell[38,39] that Krebs-cycle enzymes themselves should be active in transport by group-translocating reactions cannot be correct. This is also supported by the finding of KAY AND KORNBERG[44] of mutants that lack certain permeases but still possess the enzymes necessary to convert the substrates. At least some other proteins are also necessary, perhaps the so-called 'binding proteins'[45].

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