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THE TRANSPORT OF KREBS-CYCLE INTERMEDIATES IN *AZOTOBACTER VINELANDII* UNDER VARIOUS METABOLIC CONDITIONS

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SUMMARY

1. The oxidation of Krebs-cycle intermediates in *Azotobacter vinelandii* is inhibited by the uncouplers carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and carbonyl cyanide *m*-chlorophenylhydrazone, and energy-transfer inhibitors such as oligomycin, *N,N'*-dicyclohexylcarbodiimide or Dio-9.

2. Uncouplers and energy-transfer inhibitors have a synergistic inhibitory effect.

3. Under aerobic conditions no rapid exchange diffusion of C_4-dicarboxylates is observed.

4. Cells retain their accumulated metabolites under anaerobic conditions. Under these conditions uncouplers or energy-transfer inhibitors induce an efflux of metabolites.

5. Under anaerobic conditions exchange of intracellular for extracellular dicarboxylates is observed.

6. It is concluded from these results that in *A. vinelandii* Krebs-cycle intermediates are transported at the expense of energy. There are strong indications for a direct involvement of ATP in this energy-requiring process.

INTRODUCTION

Accumulation of Krebs-cycle intermediates by bacteria against an electrochemical gradient requires, by definition, the input of energy. However, the coupling between transport and energy supply is still a matter of debate (for a review see ref. 1). According to Kedem[2] we may speak about active transport when there is an interaction between the flow of a substrate across the membrane and a metabolic reaction. In this case there is no chemical change of the substrate during the transport process and the connection between the two processes can be very remote. Another type of transport is designated group translocation[3], in which the solute is converted during the transport process. An example is the transport of several sugars in bacteria, yielding intracellular sugar phosphates[4].

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

Little is known about the energetics of the transport of Krebs-cycle intermediates in bacteria, although it has been demonstrated that these anions can be accumulated against a concentration gradient both in intact cells[5,6] and bacterial vesicles[7,8]. The study of this process is complicated, at least in *Azotobacter vinelandii*, by the fact that substrates, once accumulated, are oxidized rapidly *via* the Krebs cycle. We have shown, however, in an earlier paper[9] that the accumulation of di- and tricarboxylates in *A. vinelandii* is inhibited by uncouplers of oxidative phosphorylation. A similar dependence on energy supply has been reported for anion transport in *Bacillus subtilis*[5,10], *Pseudomonas*[11], *Escherichia coli*[6] and *Aerobacter aerogenes*[12].

We have investigated the effect of uncouplers and inhibitors of oxidative phosphorylation on the accumulation of Krebs-cycle intermediates in somewhat more detail, in order to be able to compare it with results obtained with sugar and amino acid uptake in bacteria (*cf.* ref. 13). In particular we have investigated the possible role of exchange diffusion in *A. vinelandii*, since it has been reported that in other bacteria exchange of endogenous for exogenous anions can be observed[5,11,14] under certain conditions, comparable perhaps to the process in mitochondria[15,16]. This may teach us something about the coupling between transport and energy supply and mutual coupling between fluxes.

METHODS

A. vinelandii (strain ATCC 478) was grown, harvested and washed as described in ref. 9.

The rate of oxygen uptake was measured polarographically at 25 °C with a Clark electrode in a medium containing 25 mM Tris–HCl, 10 mM KCl, 0.5 mM EDTA and protein (about 0.3 mg/ml), final pH 7.6. The reaction was started by addition of the substrate.

Oxidative phosphorylation in intact cells was measured at 25 °C essentially as described in ref. 17. If inhibitors were present, cells were preincubated for 1 min with the compound before addition of oxygen.

Fumarate and L-malate were determined according to Schoner[18]. Pyruvate, oxaloacetate and citrate were determined according to Williamson and Corkey[19].

Exchange (diffusion) under anaerobic conditions

Cells were incubated in a vessel, containing a Clark electrode and an inlet to blow nitrogen over the solution, in a medium containing 25 mM Tris–HCl, 10 mM KCl, ^{14}C-labelled substrate and 1 mM EDTA if required, final pH 7.6. The concentration of oxygen was monitored with the Clark electrode. Upon anaerobiosis, nitrogen (oxygen content 3 ppm) was blown over the solution to keep it anaerobic. Samples were taken with a syringe and centrifuged quickly in a Coleman microcentrifuge as described in ref. 9. Additions were made anaerobically by flushing the solution with nitrogen before addition.

Chemicals

[1,4-$^{14}C_2$]Succinate, [2,3-$^{14}C_2$]succinate, [1,4-$^{14}C_2$]fumarate, [2,3-$^{14}C_2$]fumarate and ^3H_2O were obtained from the Radiochemical Centre, Amersham.

All purified enzymes were obtained from Boehringer und Soehne. *N,N'*-dicyclo-

hexylcarbodiimide (DCCD) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were obtained from Koch–Light Laboratories and Sigma, respectively.

The following compounds were gifts: Dio-9, Gist-Brocades N.V.; oligomycin, Upjohn Chem. Co.; valinomycin and nigericin, Eli Lilly and Comp.; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Dr P. Heytler.

RESULTS

Effect of uncouplers and inhibitors on oxidation in intact cells

Fig. 1 shows that preincubation of succinate-grown cells with 1.0 μM FCCP leads to complete inhibition of the succinate oxidation. The same concentration of FCCP added after the oxidation has reached its steady-state value has much less effect. Addition of FCCP at intermediate rates of oxidation leads to different percentages of inhibition (Fig. 1).

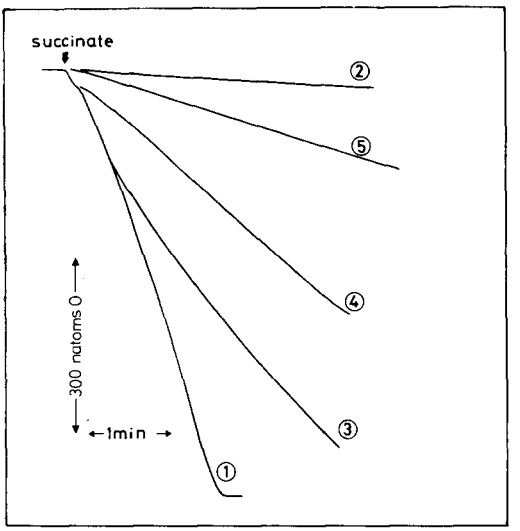
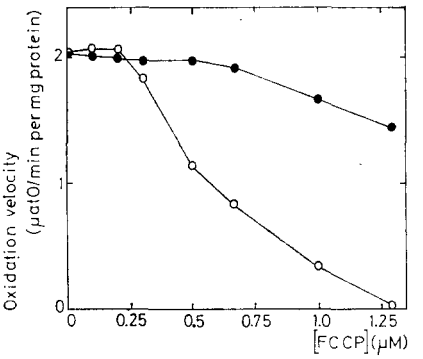


Fig. 1. Effect of FCCP on the oxidation of succinate by succinate-grown cells. Succinate-grown cells were incubated in a medium containing 25 mM Tris–HCl, 0.5 mM EDTA and 6 mM KCl, final pH 7.6. The reaction was started by the addition of 6 mM potassium succinate. The oxygen consumption was measured as described in Methods. Curve 1, no further addition; Curve 2, cells were preincubated for 1 min with 1.0 μM FCCP; Curve 3, 4 and 5, 1.0 μM FCCP was added 0 s, 10 s and 30 s, respectively, after the substrate.

Fig. 2 shows the oxidation rate as a function of the FCCP concentration (1 min preincubation). In the absence of EDTA little inhibition is observed, presumably because the cell wall is impermeable to FCCP[20]. Similar results have been obtained with other Krebs-cycle intermediates, such as fumarate, L-malate, citrate and 2-oxoglutarate, suggesting that the transport of these anions across the cytoplasmic membrane requires energy.

The oxidation of succinate can also be inhibited by inhibitors of oxidative phosphorylation, such as oligomycin, Dio-9 (Fig. 3) or DCCD (not shown). 1 min



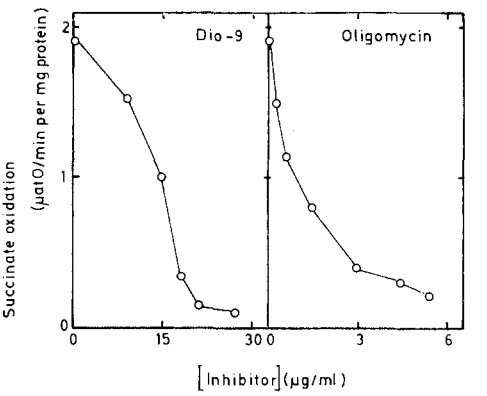


Fig. 2. Succinate oxidation as a function of the FCCP concentration. Succinate-grown cells (0.16 mg protein/ml) were incubated in a medium containing 25 mM Tris–HCl and 10 mM KCl, final pH 7.6. Oxidation was started by the addition of 6 mM succinate. If required, 1 mM EDTA was added. Cells were preincubated for 1 min with the concentration FCCP indicated. ○—○, succinate *plus* EDTA; ●—●, succinate.

Fig. 3. Effect of Dio-9 and oligomycin on the succinate oxidation. Succinate-grown cells (0.3 mg protein/ml) were preincubated for 2 min with the inhibitor in a medium containing 25 mM Tris–HCl, 10 mM KCl and 0.5 mM EDTA, final pH 7.6. Oxidation was started by the addition of 6 mM succinate.

preincubation with 66 μM DCCD leads to 86% inhibition of succinate oxidation. This is in contrast, for instance, to the aerobic proline uptake in *E. coli*, which is almost completely insensitive to DCCD even after several hours preincubation, as reported by Klein and Boyer[13]. Although the precise mechanism by which these compounds inhibit bacterial oxidative phosphorylation is not known (for a review see refs 1 and 21), several results suggest that they act directly on the ATP synthesis. Table I shows that in *A. vinelandii* DCCD, Dio-9 and FCCP indeed inhibit ATP synthesis in intact cells (higher concentrations of the various inhibitors are required in these experiments, because the protein concentration was higher). From these results it can be concluded that ATP itself is involved in the transport of Krebs-cycle intermediates in *A. vinelandii*. Uncouplers cannot release the inhibition of the succinate oxidation by inhibitors like oligomycin or Dio-9.

An interesting result is obtained when inhibitors like oligomycin and uncouplers like FCCP or CCCP are added together. Fig. 4A shows that succinate oxidation is nearly maximal in the presence of a sub-optimal concentration of Dio-9 (Curve 1). CCCP added during the steady-state oxidation has very little effect. However, cells preincubated with the same sub-optimal concentration of Dio-9 as before now become inhibited almost completely after addition of CCCP. The same synergistic effect can be observed with DCCD (Fig. 4B) or oligomycin (not shown) instead of Dio-9. This behaviour is observed only with energy-transfer inhibitors. Neither fluoroacetate, an inhibitor of the Krebs cycle, nor HQNO, an inhibitor of the respiratory chain, behave like Dio-9 or oligomycin.

Although it could be argued that these inhibitors have indirect effects on the oxidation *via* the Krebs cycle, two observations make this unlikely. First, glucose oxidation is not inhibited by these compounds, a result also found in *E. coli* (Postma,

TABLE I

EFFECT OF UNCOUPLERS AND INHIBITORS ON THE ATP SYNTHESIS IN INTACT *AZOTOBACTER VINELANDII*

Sucrose-grown cells were incubated anaerobically in the absence or presence of different inhibitors or uncouplers, in a medium containing 25 mM Tris–HCl, 10 mM KCl and 2 mM EDTA, final pH 7.6. ATP synthesis upon addition of a small amount of oxygen was measured as described in ref. 17. Temp., 25 °C.

	Inhibitor or uncoupler	*ΔATP (nmoles/mg protein)*
Expt 1	—	3.3
	3 μM FCCP	0.8
	10 μM FCCP	0.2
Expt 2	—	2.6
	50 μg/ml Dio-9	2.4
	150 μg/ml Dio-9	0.9
Expt 3	—	3.1
	200 μM DCCD	3.1
	230 μM DCCD	1.4
	260 μM DCCD	0.6
	300 μM DCCD	0.3

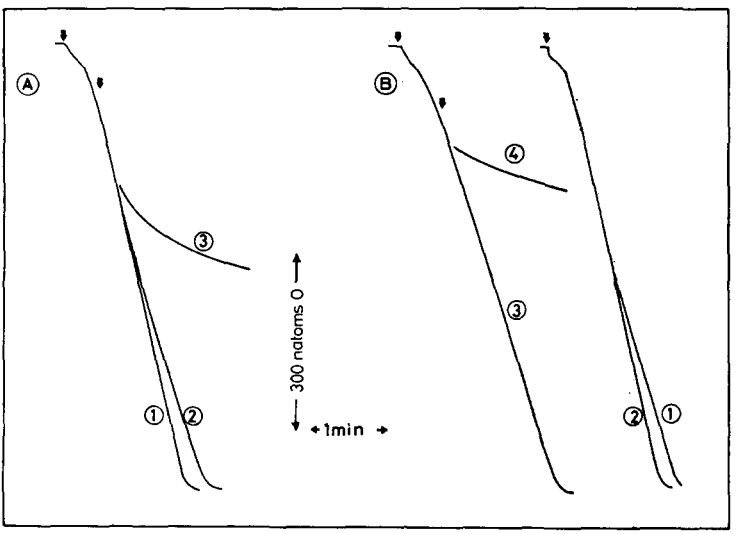


Fig 4. Effect of uncouplers and inhibitors on the succinate oxidation by intact cells. Succinate-grown cells were incubated in a medium containing 25 mM Tris–HCl, 10 mM KCl, 0.5 mM EDTA and 6 mM succinate. The reaction was started by the addition of the substrate. (A) Curve 1, cells preincubated for 1 min with 13 μg/ml Dio-9; Curve 2, CCCP (1.3 μM) added 30 s after succinate; Curve 3, cells preincubated for 1 min with 13 μg/ml Dio-9, after 30 s 1.3 μM CCCP was added. (B) Curve 1, no addition; Curve 2, CCCP (1.3 μM) was added 10 s after succinate; Curve 3, cells preincubated for 1 min with 30 μM DCCD; Curve 4, cells preincubated for 1 min with 30 μM DCCD, 1.3 μM CCCP added 10 s after succinate.

P. W., unpublished results). Furthermore, inhibitors, such as DCCD or Dio-9, as well as uncouplers, such as FCCP, inhibit ATP synthesis as measured in intact cells but do not inhibit oxidation of the endogenous NADH.

Exchange diffusion under aerobic conditions

Fig. 5 shows the inhibition of succinate oxidation by fluoroacetate. In the presence of 0.6 mM fluoroacetate, succinate cannot be oxidized, even partly, to malate or pyruvate, followed by an efflux of these anions in exchange for succinate, as occurs, for instance, in mitochondria oxidizing succinate in the presence of rotenone[22]. In our case, one should expect a maximum inhibition of 86%, 71% and 71% with L-malate, oxaloacetate and pyruvate, respectively (depending on the number of remaining oxidation steps), as endproducts of the succinate oxidation, since for the complete oxidation of succinate to CO_2 and water[9,26] 7 oxygen atoms are required.

Because even at high fluoroacetate concentrations some oxygen consumption remains (see Fig. 5), we have collected in Table II data on the excretion of C_4-

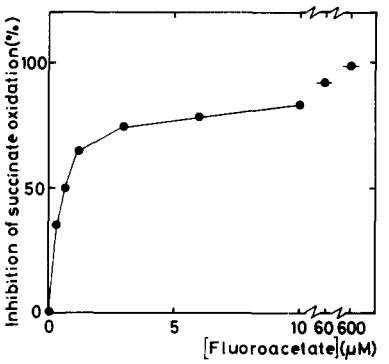


Fig. 5. Inhibition of the succinate oxidation by fluoroacetate. Succinate-grown cells were pre-incubated for 1 min in a medium containing 25 mM Tris–HCl, 10 mM KCl and fluoroacetate, final pH 7.6. The reaction was started by the addition of 6 mM succinate. Oxygen consumption was measured as described in Methods.

dicarboxylates, pyruvate and citrate in the medium during consumption of a certain amount of oxygen by succinate-grown cells or by cells adapted both to succinate and citrate. It is clear that the remaining oxygen consumption can not be due to oxidation of succinate, followed by exchange for its oxidation products, but is probably caused by a slow functioning of the Krebs cycle, since fluorocitrate, the product of fluoroacetate, is a competitive inhibitor of aconitate hydratase[23]. Earlier we have reported evidence[20] which suggests that fluoroacetate is neither an inhibitor of the dicarboxylate translocator nor prevents the movement of pyruvate in *A. vinelandii*. These results suggest that, at least under aerobic, high-energy conditions, exchange of intracellular and extracellular anions does not occur, even when these anions can, in principle, move on two different translocators. The fact that fluoroacetate also inhibits when it is added after the oxidation has reached its steady-state rate, shows furthermore that the inhibition of the oxidation is not due to lack of energy required for transport.

TABLE II

EFFLUX OF KREBS-CYCLE INTERMEDIATES DURING OXIDATION OF SUCCINATE BY FLUOROACETATE-INHIBITED CELLS

Cells (succinate-grown or citrate–succinate-adapted cells[9]), equivalent to approximately 0.5 mg protein, were preincubated for 2 min with fluoroacetate at the indicated concentration in a medium containing 25 mM Tris–HCl and 10 mM KCl, final pH 7.6. The reaction was started by the addition of 6 mM succinate. Oxygen uptake was monitored with a Clark electrode. After a certain time interval, allowing the oxidation of about 400 natoms oxygen, 1-ml samples were withdrawn and quickly centrifuged for 1 min in an Eppendorf microcentrifuge. In the supernatant Krebs-cycle intermediates were determined as described in Methods.

Cells	*Fluoro-acetate* (μM)	O_2 *uptake* (*natoms O*)	*Krebs-cycle intermediates in medium* (*nmoles*)				
			Fumarate	L-*Malate*	*Oxalo-acetate*	*Pyruvate*	*Citrate*
Succinate	6	390	0	1.5	9.5	0	6.4
Succinate	12	365	0	6.3	8.0	0	4.8
Succinate–citrate	—	308	4.5	3.0	3.0	5.1	1.0
Succinate–citrate	6	470	0	0	0	6.8	8.8

Exchange diffusion under anaerobic conditions

Direct measurement of exchange diffusion under aerobic conditions is difficult, since mutants blocked in the Krebs cycle are not available in the case of *A. vinelandii*. Also, no inhibitors are known which inhibit completely. The results reported above with fluoroacetate suggest, although indirectly, that exchange diffusion does not occur during metabolism. This is in contrast with results obtained by Lawford and Williams[14] and Willecke and Pardee[5], who presented data showing that in *Pseudomonas* and *Bacillus subtilis*, respectively, externally added tricarboxylates could replace intracellular tricarboxylates. We have tried to measure the same phenomenon under anaerobic conditions. *A. vinelandii* is unable to convert glucose anaerobically into lactate, excluding generation of energy under these conditions. Furthermore, we have shown in an earlier publication[17] that, due to the high ATPase activity, a few seconds after anaerobiosis only a low level of ATP remains.

Fig. 6 shows that metabolites are accumulated in the cell during the aerobic period when fumarate is the substrate (*cf.* ref. 9). After anaerobiosis no further accumulation occurs but, on the other hand, also no efflux of metabolites is observed for at least 10 min. No oxidation occurs due to, for instance, diffusion of oxygen; this is shown by the following facts. First, no label disappears from the medium under these conditions for at least 10 min (Fig. 6). One can calculate that, in that case, less than 1% of the oxidation rate, determined under aerobic conditions, is left. Secondly, experiments carried out in closed centrifuge tubes (*i.e.* without transferring samples from the oxygraph vessel to the tubes) give the same results.

Fig. 6 shows that addition of an excess of unlabelled dicarboxylic acid anions, such as fumarate, induces an efflux of labelled material from the succinate-grown cells, loaded with labelled fumarate. The same results can be obtained with dicarboxylates, like L-malate or succinate, but not with other Krebs-cycle intermediates,

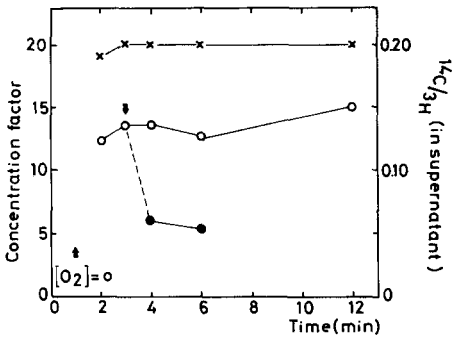


Fig 6. Exchange of dicarboxylates under anaerobic conditions. Succinate-grown cells were incubated in a medium containing 25 mM Tris–HCl, 10 mM KCl and 0.5 mM [U-^{14}C]fumarate, final pH 7.6. Accumulation after anaerobiosis was measured as described in Methods. At the arrow 5 mM KCl (○—○), or 5 mM fumarate (●—●) was added anaerobically. × — ×, concentration of labelled fumarate in the supernatant, in the experiment where KCl was added.

TABLE III

EXCHANGE OF INTRACELLULAR METABOLITES AGAINST KREBS-CYCLE INTERMEDIATES

Succinate-grown cells were loaded in a medium containing 25 mM Tris–HCl, 10 mM KCl and 0.5 mM [U-^{14}C]fumarate, final pH 7.6. 100 s after anaerobiosis, 5 mM of an anaerobic solution of different Krebs-cycle intermediates was added. Samples were taken before and after addition of the intermediates. The intracellular accumulation is expressed as the concentration factor, defined in ref. 9.

Addition	*Concentration factor*	
	Before addition	*After addition*
—	27	29
Fumarate	29	9
Succinate	26	10
L-Malate	32	5
Pyruvate	22	23
Citrate	21	22
2-Oxoglutarate	23	26

such as citrate or 2-oxoglutarate, or with pyruvate (Table III). Dicarboxylic acid anions, such as mesotartrate, L-(−)-tartrate or citramalate, that do not interact with the C_4-dicarboxylate translocator (as judged by the fact that they do not inhibit succinate oxidation) are unable to induce this efflux. The non-metabolizable analogue dihydroxyfumarate, which is transported by the dicarboxylate translocator[24], behaves like succinate or fumarate.

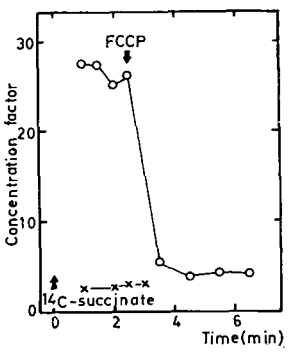
We were unable to measure the rate of this process since it was completed within 10 s, the shortest reaction period possible in our experiments. This result means that the rate of exchange is greater than 45 nmoles/min per mg protein (calculated from Fig. 6). In the experiments described in Table II, maximally 9 nmoles

Krebs-cycle intermediates/min per mg protein appear in the external medium, which gives a quantitative foundation for the statement that under aerobic conditions the exchange diffusion is relatively slow. Since in the experiments reported above the extracellular concentration is changed upon addition of the unlabelled anion, we have performed also the experiment shown in Fig. 7. Cells were loaded aerobically with unlabelled succinate. After anaerobiosis, labelled fumarate (less than 5 μM) was added. We observe that dicarboxylic acid anions can be taken up, presumably in exchange for intracellular dicarboxylates. The uptake is much less in succinate-grown cells preincubated without a substrate. Fig. 7 shows, in addition, that in sucrose-grown cells almost no dicarboxylic acid anions are accumulated under the same conditions, excluding nonspecific binding to, for instance, cationic binding sites in the membrane.

The experiment of Fig. 7 is complementary to the one shown in Fig. 6, because both figures together show that under exchange conditions compounds indeed move both inwards and outwards.

Finally, in Fig. 8 it is shown that fluoroacetate, in a concentration which inhibits the oxidation completely, does not inhibit the exchange of dicarboxylates.

The results presented in this section suggest that under anaerobic conditions a steady state exists in which influx and efflux of anions can take place. This exchange must be strictly coupled since there is a constant level of metabolites in the cell (Fig. 6). To determine whether energy is involved in this process, we have tested the effect of uncouplers on this exchange.



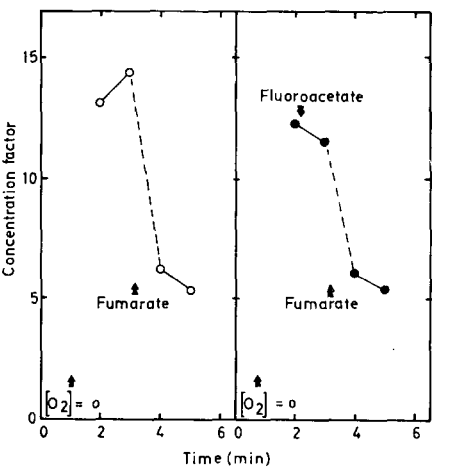


Fig 7. Exchange of dicarboxylates in cells loaded with unlabelled succinate. Succinate-grown cells were loaded in a medium containing 25 mM Tris–HCl, 10 mM KCl, 0.5 mM EDTA and 0.1 mM succinate, final pH 7.6. Final volume, 3 ml. After anaerobiosis, 0.25 μCi [U-^{14}C]succinate was added at the first arrow. At $t=2.5$ min, 10 μM FCCP was added. In the case of sucrose-grown cells no substrate was added in the medium which was made anaerobic by flushing with nitrogen. ○—○, succinate-grown cells; ×—×, sucrose-grown cells.

Fig. 8. Exchange in the absence and presence of fluoroacetate. Succinate-grown cells were loaded in a medium containing 25 mM Tris–HCl, 10 mM KCl, 0.5 mM [U-^{14}C] fumarate, final pH 7.6. 3 mM fluoroacetate was added after anaerobiosis, where indicated. Exchange was induced by the anaerobic addition of 5 mM fumarate.

Effect of uncouplers and inhibitors of oxidative phosphorylation

Fig. 9 shows that an uncoupler like FCCP induces an efflux of label when added during the anaerobic phase, in cells loaded aerobically with succinate. This efflux represents a net loss from the cell, since there was no change in the specific activity of the various metabolites under these conditions. The concentration of FCCP needed for maximal efflux is about 0.5–1.0 μM, the same concentration as that needed for the inhibition of succinate oxidation (Fig. 2). Since it could be argued that loss of intracellular compounds which are negatively charged is due to leak of potassium from the cell (as observed in mitochondria[25]), we repeated the experiment reported in Fig. 9 with valinomycin or nigericin instead of FCCP. Neither antibiotic could induce an efflux from anaerobic cells, although we know that valinomycin induces a rapid efflux of potassium while nigericin releases potassium from the cell coupled to a proton influx[26]. We have reported earlier[26] that neither valinomycin nor nigericin is able to inhibit succinate oxidation, in contrast to results obtained with uncouplers.

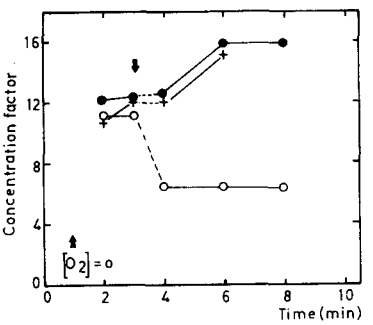


Fig. 9. Effect of uncoupler or ionophores on the accumulated dicarboxylates. Succinate-grown cells were loaded in a medium containing 25 mM Tris–HCl, 10 mM KCl, 1 mM EDTA and 0.5 mM [U-^{14}C]succinate, final pH 7.6. After anaerobiosis uncoupler or the antibiotic was added at the arrow. ○—○, 5 μM FCCP; ●—●, 3 μg/ml nigericin; +—+, 3 μg/ml valinomycin.

It can be seen from Figs 6 and 9 that only part of the accumulated metabolites can be exchanged or is able to leave the cell in the presence of excess substrate or uncoupler, respectively. Fig. 7 shows that under conditions in which cells are pre-loaded with an unlabelled dicarboxylate and subsequently brought in contact with a labelled dicarboxylate, uncouplers induce an efflux of almost all label accumulated previously. We found that a considerable part of the metabolites accumulated during oxidation of Krebs-cycle intermediates in *A. vinelandii* is citrate and glutamate, as reported previously for *E. coli*[27] and *B. subtilis*[5]. We have reported elsewhere[24] that glutamate is not permeant in *A. vinelandii*, as judged by oxidation studies, while in succinate-grown cells the tricarboxylate translocator is absent[9]. In cases where we load cells first with unlabelled metabolites and then introduce label by an exchange diffusion process, it is understandable that upon addition of an uncoupler almost all label leaks out again, since then only permeant anions are labelled.

Since inhibitors, such as Dio-9, oligomycin or DCCD, inhibit the succinate oxidation, suggesting an involvement of ATP in transport, we tested also these compounds on the exchange under anaerobic conditions. All inhibitors induced a net efflux from the cell, similar to FCCP (not shown).

DISCUSSION

The results presented in this paper suggest that the transport of Krebs-cycle intermediates in *A. vinelandii* and their subsequent metabolism are connected in such a way that the transport process fits the definition by Kedem[2] of active transport. The data can be discussed only in a qualitative manner, however, since the measurement of rate constants or velocities has been impossible.

In recent years the transport of sugars and amino acids has been investigated extensively in bacteria to establish the qualitative relation between transport and energy supply. Several proposals have been advanced concerning the nature of the primary energy donor: ATP[28,29], a certain conformation of the membrane[13], a membrane potential or a pH gradient[30–33] or direct coupling of the translocation with the electron transport chain[34]. It has been shown, furthermore, that some sugars are transported at the expense of phosphoenolpyruvate (*cf.* ref. 3). In the presence of uncouplers or metabolic inhibitors only equilibration of the internal and external metabolites can occur, a process called facilitated diffusion (*cf.* ref. 35). Recently, however, Koch[36] published an article on the transport of galactosides in *E. coli*, in which he suggested that even transport down the electrochemical gradient may require energy, since in completely starved cells uncouplers inhibit hydrolysis of nitrophenyl galactoside (earlier results[37,38] indicating the opposite may be attributed to incomplete starvation[36]).

Superficially, the results obtained by us with Krebs-cycle intermediates look similar to those obtained with galactosides, for instance. Two distinct differences should be emphasized.

(i) Even in the absence of energy-generating metabolism (during anaerobiosis), a steady-state level of intracellular metabolites is maintained against a concentration gradient in *A. vinelandii* (*cf.* Fig. 6). Only after the addition of an uncoupler (Fig. 9) or an inhibitor of oxidative phosphorylation does net efflux occur.

(ii) Uncouplers or inhibitors of oxidative phosphorylation are able to inhibit the oxidation of Krebs-cycle intermediates (Fig. 1, Curve 2). This suggests that rapid facilitated diffusion is not possible under these conditions, otherwise oxidation of succinate should proceed at an extracellular concentration of 6 mM.

Under anaerobic conditions Krebs-cycle intermediates can be taken up in exchange for intracellular anions (Figs 6 and 7). Since the flux leads to accumulation of the ions and thus requires energy, this flux must be coupled to the outwardly directed flux in such a way that one flux drives the other. Uncouplers are apparently able to dissociate both fluxes from each other, possibly by discharging a high-energy state generated by the efflux of an intracellular compound. Coupling of fluxes has been proposed also by Tanner and co-workers[39,40] for hexose transport in *Chlorella*. They also observed that cells did not lose their contents whenever there was a lack of energy, but in these experiments even uncouplers did not induce an efflux of the accumulated sugars.

A second interesting observation is the inhibition of the oxidation by inhibitors of ATP synthesis, like oligomycin, Dio-9 or DCCD. This suggests that ATP is in some way involved in transport, an observation also made by Scarborough *et al.*[28] for galactoside transport in *E. coli* and by Knape *et al.*[29] for arginine transport in *E. coli*. Although we do not know whether these inhibitors act specifically on the

ATP synthesizing complex in intact *A. vinelandii*, Table I shows that these inhibitors at least inhibit ATP synthesis. Furthermore, in *E. coli* succinate oxidation is also inhibited by FCCP and DCCD (Postma, P. W., unpublished observations), while in that case it is known that DCCD inhibits the ATPase in intact cells and extracts[41,42] and FCCP uncouples oxidative phosphorylation[41].

Although ATP could act either by direct interaction with the translocator or by generating a membrane potential or pH difference which can drive the transport, the results obtained with inhibitors like oligomycin suggest that the second possibility is rather unlikely since exchange diffusion is inhibited both by uncouplers and inhibitors.

To illustrate our interpretation of the data we may use the scheme outlined in Fig. 10. In this scheme it is assumed that the translocator can return empty, after having transported substrates into the cell at the expense of ATP (Fig. 10A). In Fig. 10B is shown the case of exchange diffusion when the internal concentration is higher than the concentration in the medium for substrate 1 ($S_i^1 > S_o^1$), while the reverse is true for the other substrate. Although it is suggested here that only one type of translocator is involved in this process, it could equally well be that energy generated by the outward movement of a dicarboxylic acid anion on its translocator can provide the energy for the inward movement of, for instance, a tricarboxylic acid anion on another translocator.

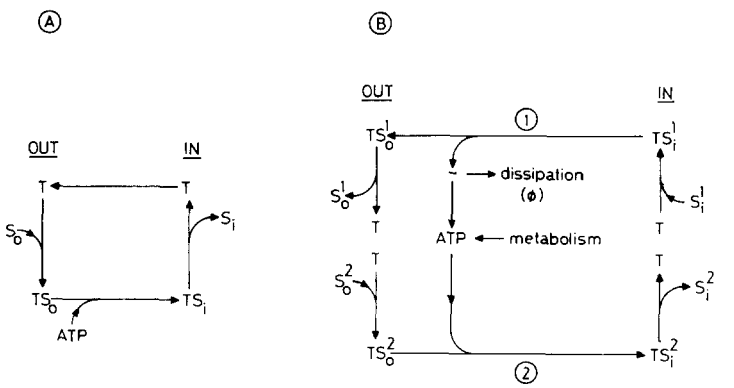


Fig 10. Model for the transport of Krebs-cycle intermediates in *Azotobacter vinelandii*.

A consequence of the model is that one has to assume that the ATP, involved in transport, is not in rapid connection with the ATP pool measured during the ATPase in intact cells, since we know that intact cells contain a high ATPase activity[17], while on the other hand the steady-state level of intracellular metabolites can be maintained for at least 10 min. Although it makes sense physiologically that cells do not lose their intracellular components as soon as they reach a low-energy state, as suggested already by Komor *et al.*[39], it is difficult to combine these findings in a satisfactory model.

Results obtained with transport of Krebs-cycle intermediates in other bacteria show that accumulation is sensitive to uncouplers[5,6,11,12], but in these cases accumulation against a gradient under aerobic conditions was measured.

Rayman *et al.*[7] showed that membrane vesicles of a mutant of *E. coli*, deficient

in succinate dehydrogenase and fumarate hydratase, could accumulate Krebs-cycle intermediates in the presence of an oxidizable substrate, as shown earlier by Kaback and co-workers for sugars and amino acids (*cf.* ref. 43). Rayman *et al.*[7] concluded that oxidative phosphorylation is not involved in transport in vesicles (as stated earlier also by Kaback[43] and recently by Klein and Boyer[13]) because oligomycin does not inhibit and ATP can not stimulate the transport. This conclusion, however, is not well founded, since (i) oligomycin does not inhibit the ATPase in *E. coli* (*cf.* refs 21 and 42) and (ii) up until now it has not been shown conclusively that ATP can reach its natural site of action in bacterial vesicles which are oriented in such a way that they are capable of accumulating metabolites. Although studies by Klein and Boyer[13] suggest that either oxidation or ATP may serve as energy source for transport of amino acids in intact *E. coli* cells by generating some high-energy state in the membrane, the results obtained by us with *A. vinelandii* favour the interpretation that ATP is directly involved in the transport of Krebs-cycle intermediates.

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REFERENCES

1 Harold, F. M. (1972) *Bacteriol. Rev.* 36, 172–230
2 Kedem, O. (1961) in *Membrane Transport and Metabolism* (Kleinzeller, A. and Kotyk, A., eds), pp. 87–93, Academic Press, New York
3 Mitchell, P. (1967) *Adv. Enzymol.* 29, 33–87
4 Roseman, S. (1969) *J. Gen. Physiol.* 54, 138S–184S
5 Willecke, K. and Pardee, A. B. (1971) *J. Biol. Chem.* 246, 1032–1040
6 Lo, T. C. Y., Rayman, M. K. and Sanwal, B. D. (1972) *J. Biol. Chem.* 247, 6323–6331
7 Rayman, M. K., Lo, T. C. Y. and Sanwal, B. D. (1972) *J. Biol. Chem.* 247, 6332–6339
8 Murakawa, S., Izaki, K. and Takahashi, H. (1971) *Agric. Biol. Chem.* 35, 1992–1993
9 Postma, P. W. and van Dam, K. (1971) *Biochim. Biophys. Acta* 249, 515–527
10 Fournier, R. E., McKillen, M. N., Pardee, A. B. and Willecke, K. (1972) *J. Biol. Chem.* 247, 5587–5595
11 Lawford, H. G. (1971) *The Transport of Citrate and Other Tricarboxylic Acids in Pseudomonas fluorescens*, Thesis, Toronto
12 Wilkerson, L. S. and Eagon, R. G. (1972) *Arch. Biochem. Biophys.* 149, 209–221
13 Klein, W. L. and Boyer, P. D. (1972) *J. Biol. Chem.* 247, 7257–7265
14 Lawford H. G. and Williams, G. R. (1971) *Biochem. J.* 123, 571–577
15 Chappell, J. B. (1968) *Br. Med. Bull.* 24, 150–157
16 Klingenberg, M. (1970) *Essays Biochem.* 6, 119–159
17 Baak, J. M. and Postma, P. W. (1971) *FEBS Lett.* 19, 189–192
18 Schoner W. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), 2. Auflage, Band II, pp. 1551–1555, Verlag Chemie, Weinheim/Bergstr.
19 Williamson, J. R. and Corkey, B. E. (1969) *Methods Enzymol.* 13, 434–513
20 Visser, A. S. and Postma, P. W. (1973) *Biochim. Biophys. Acta* 298, 333–340
21 Harold, F. M. (1970) *Adv. Microbial Physiol.* 4, 45–104
22 Chappell, J. B. and Haarhoff, K. N. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z. and Wojtczak, L., eds), pp. 75–91, Academic Press, New York
23 Morrison, J. F. and Peters, R. A. (1954) *Biochem. J.* 58, 473–479

24 Reuser, A. J. J. and Postma, P. W. (1973) *Eur. J. Biochem.* 33, 584–592
25 Kimmich, G. A. and Rasmussen, H. (1967) *Biochim. Biophys. Acta* 131, 413–420
26 Postma, P. W., Visser, A. S. and van Dam, K. (1973) *Biochim. Biophys. Acta* 298, 341–353
27 Kay, W. W. and Kornberg, H. L. (1971) *Eur. J. Biochem.* 18, 274–281
28 Scarborough, G. A., Rumley, M. K. and Kennedy, E. P. (1968) *Proc. Natl. Acad. Sci. U.S.* 60, 951–958
29 Knape, H., Boquet, P. L. and Röschenthaler, R. (1972) *FEBS Lett.* 19, 311–314
30 Pavlasova, E. and Harold, F. M. (1969) *J. Bacteriol.* 98, 198–204
31 West, I. and Mitchell, P. (1972) *Bioenergetics* 3, 445–462
32 Schairer, H. U. and Haddock, B. A. (1972) *Biochem. Biophys. Res. Commun.* 48, 544–551
33 Kashket, E. R. and Wilson, T. H. (1972) *Biochem. Biophys. Res. Commun.* 49, 615–620
34 Kaback, H. R. and Barnes, Jr, E. M. (1971) *J. Biol. Chem.* 246, 5523–5531
35 Kepes, A. (1971) *J. Membrane Biol.* 4, 87–112
36 Koch, A. L. (1971) *J. Mol. Biol.* 59, 447–459
37 Koch, A. L. (1964) *Biochim. Biophys. Acta* 79, 177–200
38 Winkler, H. H. and Wilson, T. H. (1966) *J. Biol. Chem.* 241, 2200–2211
39 Komor, E., Haass, D. and Tanner, W. (1972) *Biochim. Biophys. Acta* 266, 649–660
40 Decker, M. and Tanner, W. (1972) *Biochim. Biophys. Acta* 266, 661–669
41 Gutnick, D. L., Kanner, B. I. and Postma, P. W. (1972) *Biochim. Biophys. Acta* 283, 217–222
42 Roisin, M. P. and Kepes, A. (1972) *Biochim. Biophys. Acta* 275, 333–346
43 Kaback, H. R. (1972) *Biochim. Biophys. Acta* 265, 367–416