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PERMEABILITY OF *AZOTOBACTER VINELANDII* TO CATIONS AND ANIONS

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SUMMARY

1. The permeability of *Azotobacter vinelandii* to different cations and anions has been investigated.

2. Experiments with acetate suggest that an energy-linked potassium pump is present in the membrane of *A. vinelandii*. The membrane is not permeable to Na^+ or NH_4^+.

3. Acetate moves as the undissociated acid (or as anion in exchange for OH^-). The other Krebs-cycle intermediates and pyruvate move as the anion only.

4. The transport of succinate (and probably also of other Krebs-cycle intermediates) is energy dependent since it is inhibited by uncouplers of oxidative phosphorylation. The movement of pyruvate or acetate is not inhibited by an uncoupler.

5. Valinomycin, nigericin and monensin, as well as uncouplers, are very active in *A. vinelandii* provided the cells are pretreated with EDTA.

INTRODUCTION

Bacterial cells, like other cells, can maintain a concentration gradient of different compounds across their membrane due to the fact that the membrane is relatively impermeable for most compounds. Only by regulating the transport of substances *via* special translocators in both directions across the membrane, a steady-state level of metabolites can be maintained inside the cell, optimal for its functioning. A number of transport systems or translocators has been described both for anions and cations as well as for uncharged molecules, serving this purpose (for a review see ref. 1).

Recently we described the existence of at least four different translocators for di- and tricarboxylic acid anions that can be induced in *Azotobacter vinelandii*[2]. The movement of potentially charged molecules raises the question how electroneutrality is maintained. We demonstrated[2] that Krebs-cycle intermediates are oxidized nearly completely to CO_2 and water. This rules out the continuous operation of exchange-diffusion carriers, as described in mitochondria[3] and in bacteria by Lawford and Williams[4], under metabolic conditions in *A. vinelandii*. However, it has to be investigated whether exchange against CO_2 is possible.

Abbrevations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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Extending the observations of Knowles and Smith[5,6] we have studied the permeability to several cations and anions, using swelling as a measure, of *A. vinelandii*. In the accompanying paper[7] we will report more specifically on the movement of K^+ and H^+ in connection with metabolism and the effect of cations on translocators.

METHODS

A. vinelandii (strain ATCC 478) was grown, harvested and washed as described in ref. 2.

Protoplasts

Protoplasts of *A. vinelandii* were prepared essentially as described by Repaske[8], using a medium containing 1 mM Tris–HCl, 1 mM EDTA and 40 μg lysozyme (EC 3.2.1.17) per ml, final pH 7.6, and the required salt to stabilize the protoplasts osmotically.

Measurement of turbidity changes

Swelling and shrinkage of intact cells and protoplasts were measured by following the changes in light scattering at 680 nm using a Zeiss PMQ II spectrophotometer. Salts were added to cells, suspended in 1 mM Tris–HCl and 1 mM EDTA, final pH 7.6, to the concentrations indicated in Results and Discussion. When swelling of protoplasts was studied, cells were suspended in 1 mM Tris–HCl, 1 mM EDTA and the required salt. After 1 min equilibration, lysozyme was added.

Chemicals

Valinomycin, nigericin and monensin were a gift of Eli Lilly and Comp. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of Dr Heytler. Lysozyme was obtained from Sigma Chemical Co.

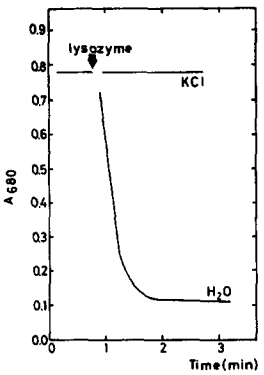
RESULTS AND DISCUSSION

Permeability to inorganic cations and anions

Recently Knowles and Smith[5] investigated the permeability to several salts of intact cells of *A. vinelandii*, showing that the change in light scattering is paralleled by changes in the volume of the bacteria. We have extended these studies, using also protoplasts.

Fig. 1 shows that 40 mM KCl is able to stabilize protoplasts. The swelling and bursting of protoplasts in water is completed in less than 1 min after the addition of lysozyme. Using this technique, it can be shown that the K^+, Na^+ and NH_4^+ salts of Cl^-, PO_4^{3-} and SO_4^{2-} are able to stabilize protoplasts, confirming the data on permeability obtained by Knowles and Smith[5], with the exception of NH_4Cl which in our hands is not permeant.

To determine whether the anion or the cation or both are responsible for the inability of the salt to permeate, we studied the behaviour of the cations in combination with anions such as CNS^-, NO_3^- or I^-. These anions are thought to be permeant anions. Furthermore, in these studies we have made use of the permeation patterns of ions, induced by several compounds as described in the literature (for a review *cf.*



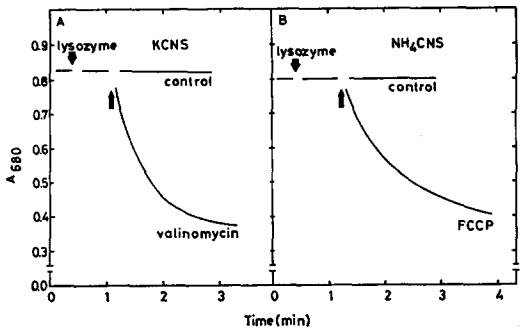


Fig. 1. Swelling of *A. vinelandii* protoplasts. Swelling was measured as described in Methods. KCl was added to a final concentration of 40 mM.

Fig. 2. Swelling of *A. vinelandii* protoplasts in different salts. Swelling was measured as described in Methods. Where indicated, valinomycin (0.3 μg/ml) or FCCP (3 μM) was added at the second arrow. KCNS and NH_4CNS were added to a final concentration of 40 mM.

ref. 9). Valinomycin and uncoupler induce an electrogenic movement of K^+ or H^+, respectively. Nigericin and monensin cause the electroneutral exchange of K^+ or Na^+, respectively, for H^+. Fig. 2 shows the behaviour of protoplasts in KCNS or NH_4CNS and the effect of valinomycin or an uncoupler like FCCP. It can be concluded that protoplasts lyse in salts of CNS^- as well as NO_3^- or I^- only when the membrane is made permeable to K^+ by valinomycin or to H^+ by an uncoupler, NH_3 being permeant. However, even in the presence of valinomycin or an uncoupler, the K^+ and NH_4^+ salts of Cl^-, SO_4^{2-} or PO_4^{3-} are still impermeant. Hamilton[10], using a similar technique, arrived at somewaht different conclusions concerning the permeability to for instance NO_3^-, NH_3 (or NH_4^+) and the effect of valinomycin in *Bacillus megaterium*. His conclusion was based primarily on the assumption that swelling in ammonium acetate is due to permeation of NH_4^+ and the acetate anion.

In contrast to the findings with protoplasts, the antibiotic valinomycin and uncouplers like FCCP were not active at comparable concentrations in cells which were shrunken in KCNS or NH_4CNS. Fig. 3 shows that addition of a low concentra-

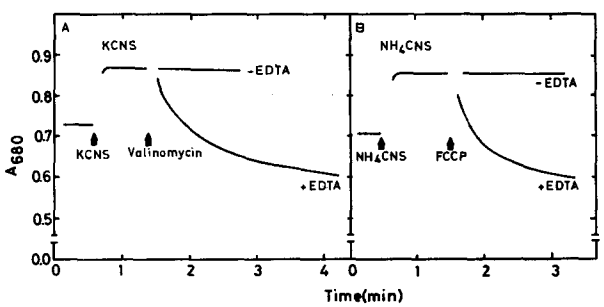


Fig. 3. Effect of EDTA on swelling induced by valinomycin or an uncoupler. Cells were incubated in a medium containing 1 mM Tris–HCl, pH 7.6, with or without 1 mM EDTA. The reaction was started by adding 0.3 ml 1 M KCNS (A) or 1 M NH_4CNS (B) to 2.7 ml medium. Where indicated valinomycin (0.3 μg/ml) or FCCP (3 μM) was added.

tion of EDTA restores the sensitivity to valinomycin and FCCP and also to nigericin (not shown). EDTA is only active when Tris is used at the same time. It has been described by Leive[11] that low concentrations of EDTA produce a non-specific increase in permeability in *Escherichia coli*. Increased permeability to uncouplers and valinomycin has been reported by Pavlasova and Harold[12] in *E. coli*. Using Tris and EDTA together, less than 0.2 μg/ml valinomycin or 0.5 μM FCCP are sufficient to render the membrane of *A. vinelandii* permeable to K^+ and H^+, respectively.

Swelling in acetate

As we published earlier[2], acetate is oxidized both by sucrose-grown and by succinate-grown cells. It is unknown whether a specific translocator is involved in acetate transport in *A. vinelandii*. Of all the metabolites tested, including all Krebs-cycle intermediates, only acetate gives rise to a rapid swelling in shrunken intact cells. Fig. 4A shows that after shrinkage sucrose-grown cells rapidly swell again in 100 mM

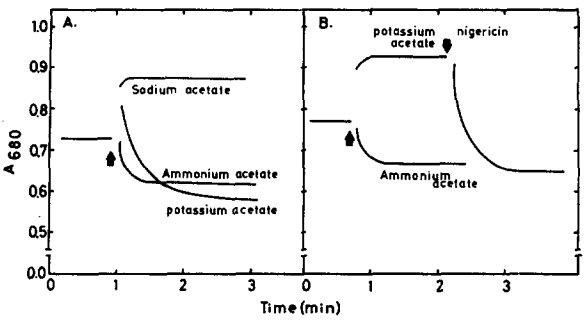


Fig. 4. Swelling of sucrose-grown *A. vinelandii* in different acetate salts. Cells were incubated in 1 mM Tris–HCl, pH 7.6, and 1 mM EDTA. Reaction was started by adding 0.3 ml 1 M acetate salt to 2.7 ml medium. A. Without arsenite. B. With 6 mM arsenite present in the incubation mixture. Where indicated nigericin (0.3 μg/ml) was added.

ammonium acetate or potassium acetate. They do not swell in sodium or Tris acetate, provided the cells are well washed with distilled water. In contrast to the findings with KCNS and NH_4CNS, this swelling is not dependent on valinomycin or an uncoupler. However, swelling in potassium acetate is inhibited when arsenite or an uncoupler is added, as shown in Fig. 4B, whereas in that case the swelling in ammonium acetate is not inhibited. This indicates that the movement of acetate or acetic acid itself is not inhibited. Oxidation studies have shown that arsenite is an inhibitor of the acetate oxidation *via* the Krebs cycle. Assuming that acetate moves as the undissociated acid, net swelling can only occur when the H^+, liberated inside, is neutralized either by permeation of NH_3 or by a K^+–H^+ exchange which for the moment we propose to be energy dependent. This model predicts that when the uptake of K^+ in exchange for H^+ is blocked by inhibition of the metabolism, nigericin, which catalyzes a K^+–H^+ exchange[13], should induce a rapid swelling. Fig. 4B shows this to be the case.

It can be shown that K^+ transport is energy dependent. Fig. 5 shows that cells can swell in sodium acetate when 1 mM KCl is added. However, arsenite-inhibited cells do not swell in 100 mM sodium acetate *plus* 1 mM KCl even after the addition of nigericin. This is to be expected because the intracellular concentration of K^+

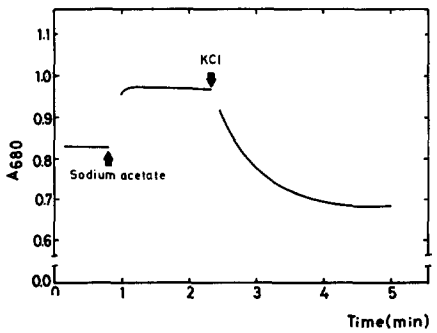


Fig. 5. Effect of K^+ on the swelling of intact cells in sodium acetate. Cells were incubated in 1 mM Tris–HCl, pH 7.6. The reaction was started by adding 0.3 ml 1 M sodium acetate to 2.7 ml medium. KCl was added to a final concentration of 1 mM.

is at least 20 mM. Nigericin cannot transport K^+ against a concentration gradient, but an energy-linked K^+ pump can. Whether the primary process is the energy-linked expulsion of protons, followed by the movement of K^+ down the electrical gradient or K^+ is first taken up at the expense of energy, followed by expulsion of protons cannot be decided here.

In addition it can be shown that the antibiotic monensin, which catalyzes a Na^+–H^+ exchange[14], is active in *A. vinelandii*. Cells suspended in 100 mM sodium acetate swell upon addition of monensin.

The results reported here suggest either that acetate moves in the undissociated form across the membrane or that the acetate anion exchanges with hydroxyl as suggested also by Mitchell and Moyle[15]. The same conclusion has been drawn for the movement of acetate in mitochondria[3]. The movement of acetate is not inhibited by an uncoupler. This result is in contrast with for instance the transport of succinate, as will be reported below. It should be remembered, however, that independent of the cation acetate is always oxidized, this means transported across the membrane.

Swelling in bicarbonate

It has been suggested in the Introduction that bicarbonate possibly acts as a counterion for Krebs-cycle intermediates. In that case one expects that cells swell in bicarbonate, although most anions like PO_4^{3-}, SO_4^{2-} and Cl^- are impermeant. Sucrose- or succinate-grown cells do indeed swell in 100 mM $KHCO_3$ but not in 100 mM $NaHCO_3$, comparable to the result obtained with acetate. Arsenite inhibits the swelling but valinomycin and, to a lesser extent, also nigericin can restore the swelling in $KHCO_3$. From this we may conclude that both HCO_3^- and CO_2 can permeate. The observation that also FCCP slightly stimulates the swelling under these conditions cannot be easily explained because this implies that the uncoupler also increases the permeability of the membrane for K^+. Low concentrations of succinate or acetate can stimulate the swelling of succinate-grown cells in $KHCO_3$ as shown in Fig. 6. This stimulation is larger than the swelling already observed in KCl *plus* a low concentration of succinate (see Fig. 6). We cannot decide in which form the carbonic acid is preferentially transported, but it seems unlikely that a strict coupling exists between substrate anion transport and transport of bicarbonate, comparable to the coupling

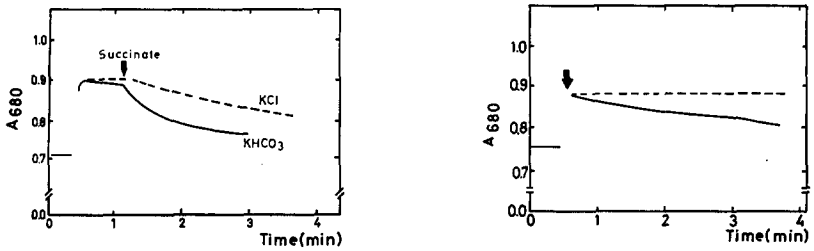


Fig. 6. Effect of succinate on the swelling of succinate-grown cells in different salts. Cells were incubated in 1 ml Tris–HCl, pH 7.6. The reaction was started by adding 0.3 ml 1 M KCl or 0.3 ml 1 M $KHCO_3$ (pH 8.0) to 2.7 ml medium. Tris–succinate was added to a final concentration of 3 mM.

Fig. 7. Swelling of succinate-grown cells in different Krebs-cycle intermediates. Succinate-grown cells were incubated in 1 mM Tris–HCl. The reaction was started by adding 0.3 ml of the different Krebs-cycle intermediates (1 M) to 2.7 ml medium. – – –, sodium succinate, potassium citrate, potassium 2-oxoglutarate, potassium malonate, potassium glutamate; ———, potassium succinate, potassium fumarate.

between phosphate and Krebs-cycle intermediates in mitochondria[3], because CO_2 produced during metabolism of sugars has to leave the cell in a neutral form.

Swelling in Krebs-cycle intermediates

Knowles and Smith[6] have studied the swelling of cells which had been shrunken in KCl, by addition of different metabolites like pyruvate or β-hydroxybutyrate. Although we can confirm their results it must be remarked that these experiments can only be performed in a medium containing some K^+. No swelling occurs in a medium containing only Na^+ or $Tris^+$ as a cation (provided well-washed cells are used). This illustrates again the important role of K^+. A similar conclusion was reached by Packer and Perry[16]. However, whether K^+, Na^+ or $Tris^+$ is the cation, oxidation of succinate is observed. Although the low concentrations of K^+ always present in the bacterial suspension may be sufficient to activate succinate oxidation (either by cycling across the membrane or by direct activation of the transport system) it is evidently not sufficient to support net salt uptake[7].

We have studied furthermore the swelling in 100 mM of different metabolites. Fig. 7 shows that succinate-grown cells swell slowly in potassium succinate or potassium fumarate but not in glutamate, 2-oxoglutarate, citrate or malonate. No swelling occurs in 100 mM sodium succinate, unless 1 mM KCl is added. Fig. 8 shows that the swelling in potassium succinate is inhibited by arsenite but not by fluoroacetate, both compounds being inhibitors of the succinate oxidation. However, arsenite inhibits under these conditions the oxidation almost 100% while in the presence of fluoroacetate some oxidation is going on, probably due to the fact that fluorocitrate is a competitive inhibitor. Addition of valinomycin has markedly different results. Fig. 8A shows that valinomycin cannot stimulate the swelling of arsenite-inhibited cells. Fig. 8B shows that the swelling of fluoroacetate-inhibited cells is stimulated by valinomycin. Nigericin has no effect under these circumstances. From these results one could conclude that succinate is transported as the anion and potassium moves with the help of valinomycin to compensate the negative charge. The difference between

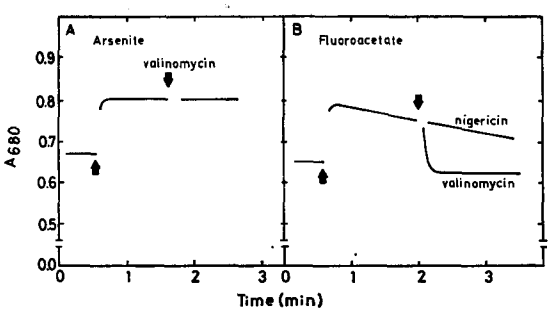


Fig. 8. Effect of inhibitors on the swelling in potassium succinate. Succinate-grown cells were suspended in a medium containing 1 mM Tris–HCl, 1 mM EDTA and 6 mM arsenite (A) or 6 mM fluoroacetate (B). Reaction was started by adding 0.3 ml 1 M potassium succinate to 2.7 ml medium. Where indicated valinomycin (0.3 μg/ml) or nigericin (0.3 μg/ml) was added.

arsenite and fluoroacetate could be interpreted to mean that the former, in addition to its inhibition of the Krebs cycle, also inhibits the dicarboxylic acid anion translocator. In that case one should expect that fluoroacetate-inhibited cells can swell in ammonium succinate upon addition of an uncoupler. This is not the case.

We should like to interpret the results with Krebs-cycle intermediates in the following way. Occurrence of swelling is determined by the balance between influx of the metabolite and removal of the metabolite or its products *via* the Krebs cycle, as pointed out also by Bovell *et al.*[17]. We will show in the accompanying paper[7] that also the oxidation of succinate and other Krebs-cycle intermediates is stimulated by K^+ and to a lesser extent by Na^+, and furthermore show that this stimulation is probably at the level of the influx.

As to energy dependence of the transport of Krebs-cycle intermediates, we have suggested earlier[2] that transport of Krebs-cycle intermediates in *A. vinelandii* is energy dependent. The results reported here support this view. Fluoroacetate-inhibited cells do not swell in ammonium succinate *plus* an uncoupler (which can be shown to inhibit the oxidation), although they do swell in potassium succinate *plus* valinomycin, proving that fluoroacetate at least does not inhibit the translocator. Possibly an uncoupler prevents the translocator to transport succinate across the membrane, even down the gradient. This is reminiscent of the conclusion by Koch[18] namely that energy is required for transport both uphill and downhill.

Another metabolite we have studied is pyruvate. It is not known whether a specific translocator is needed for this anion in *A. vinelandii*. Swelling occurs in potassium or ammonium pyruvate but not in sodium pyruvate unless KCl is added. Swelling is inhibited by arsenite or fluoroacetate. In contrast to the results with succinate, arsenite-inhibited cells swell in potassium pyruvate *plus* valinomycin or ammonium pyruvate *plus* uncoupler. These results suggest that arsenite is not inhibiting the movement of pyruvate *per se* and also that the transport of pyruvate is not inhibited by an uncoupler. Possibly pyruvate just permeates the membrane without mediation by an active translocator.

From these results it can be concluded that there is a difference between the transport of acetate on the one hand, and succinate, pyruvate and possibly bicarbonate on the other hand. The transport of acetate (potassium salt) is stimulated by nigericin,

indicating a K^+–H^+ exchange, while the transport of the other salts is stimulated by valinomycin, suggesting only a movement of potassium. Possibly acetate is transported as an acid (or in exchange for OH^-) while the other compounds are transported as anions.

Swelling of protoplasts during metabolism

Because protoplasts are much more fragile than intact cells, it was thought that swelling should be more pronounced after converting intact cells into protoplasts. It turned out, however, that only swelling of protoplasts in acetate exhibited the same phenomena as found in intact cells. This proves that the energy-dependent K^+ pump is still present. However, protoplasts made from succinate-grown cells do not swell in potassium succinate or pyruvate. Oxidation studies show that both substrates are oxidized by protoplasts although at a slower rate, proving in the case of succinate that the translocator is still present. The negative result of the swelling experiment could be caused by the fact that upon conversion of intact cells into protoplasts part of the translocator is inactivated or released since the oxidation velocity is also decreased. In that case the influx decreases but the conversion to CO_2 *via* the Krebs cycle probably not.

ACKNOWLEDGEMENTS

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REFERENCES

1 Lin, E. C. C. (1970) *Annu. Rev. Genet.* 4, 225–262
2 Postma, P. W. and van Dam K. (1971) *Biochim. Biophys. Acta* 249, 515–527
3 Klingenberg, M. (1970) *Essays Biochem.* 6, 119–159
4 Lawford, H. G. and Williams, G. R. (1971) *Biochem. J.* 123, 571–577
5 Knowles, C. J. and Smith, L. (1971) *Biochim. Biophys. Acta* 234, 144–152
6 Knowles, C. J. and Smith, L. (1971) *Biochim. Biophys. Acta* 234, 153–161
7 Postma, P. W., Visser, A. S. and van Dam, K. (1973) *Biochim. Biophys. Acta* 298, 341–353
8 Repaske, R. (1958) *Biochim. Biophys. Acta* 30, 225–232
9 Harold, F. M. (1970) *Adv. Microbial Physiol.* 4, 45–104
10 Hamilton, W. A. (1970) in *Membranes: Structure and Function* (Villanueva, J. R. and Ponz, F., eds), Vol. 20, pp. 71–79, Academic Press, London
11 Leive, L. (1965) *Proc. Natl. Acad. Sci. U.S.* 53, 745–750
12 Pavlasová, E. and Harold, F. M. (1969) *J. Bacteriol.* 98, 198–204
13 Pressman, B. C., Harris, E. J., Jagger, W. S. and Johnson, J. H. (1967) *Proc. Natl. Acad. Sci. U.S.* 58, 1949–1956
14 Pressman, B. C. (1968) *Fed. Proc.* 27, 1283–1288
15 Mitchell, P. and Moyle, J. (1956) *Symp. Soc. gen. Microbiol.* 6, 150–180
16 Packer, L. and Perry, M. (1961) *Arch. Biochem. Biophys.* 95, 379–388
17 Bovell, C. R., Packer, L. and Helgerson, R. (1963) *Biochim. Biophys. Acta* 75, 257–266
18 Koch, A. L. (1971) *J. Mol. Biol.* 59, 447–459.