Biochimica et Biophysica Acta, 766 (1984) 77–87
Elsevier

BBA 41533

ENERGETICS OF SODIUM-DEPENDENT α-AMINOISOBUTYRIC ACID TRANSPORT IN THE MODERATE HALOPHILE *VIBRIO COSTICOLA*

FRANCETTE HAMAIDE [a], G. DENNIS SPROTT [b] and DONN J. KUSHNER [a]

[a] *Department of Biology, University of Ottawa, Ottawa, K1N 6N5, and* [b] *Division of Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, K1A 0R6 (Canada)*

(Received October 10th, 1983)
(Revised manuscript received March 23rd, 1984)

Key words: Aminoisobutyric acid transport; Membrane potential; Respiration inhibitor; Halophilic bacteria; Na^+ dependence; (V. costicola)

The energetics of α-aminoisobutyric acid transport were examined in *Vibrio costicola* grown in a medium containing the NaCl content (1 M) optimal for growth. Respiration rate, the membrane potential ($\Delta\psi$) and α-aminoisobutyric acid transport had similar pH profiles, with optima at 8.5–9.0. Cells specifically required Na^+ ions to transport α-aminoisobutyric acid and to maintain the highest $\Delta\psi$ (150–160 mV). Sodium was not required to sustain high rates of O_2-uptake. $\Delta\psi$ (and α-aminoisobutyric acid transport) recovered fully upon addition of Na^+ to Na^+-deficient cells, showing that Na^+ is required in formation or maintenance of the transmembrane gradients of ions. Inhibitions by protonophores, monensin, nigericin and respiratory inhibitors revealed a close correlation between the magnitudes of $\Delta\psi$ and α-aminoisobutyric acid transport. Also, dissipation of $\Delta\psi$ with triphenylmethylphosphonium cation abolished α-aminoisobutyric acid transport without affecting respiration greatly. On the other hand, alcohols which stimulated respiration showed corresponding increases in α-aminoisobutyric acid transport, without affecting $\Delta\psi$. Similarly, *N*,*N*′-dicyclohexylcarbodiimide (10 μM) stimulated respiration and α-aminoisobutyric acid transport and did not affect $\Delta\psi$, but caused a dramatic decline in intracellular ATP content. From these, and results obtained with artificially established energy sources ($\Delta\psi$ and Na^+ chemical potential), we conclude that $\Delta\psi$ is obligatory for α-aminoisobutyric acid transport, and that for maximum rates of transport an Na^+ gradient is also required.

Introduction

A concentrative transport of nutrients across the cytoplasmic membrane can be accomplished utilizing the energy of the transmembrane electrochemical proton gradient (protonmotive force) (for reviews see Refs. 1–3). This protonmotive force is generated by the efflux of protons during respiration, hydrolysis of ATP by adenosine triphosphatase, or through photosynthetic or bacteriorhodopsin activity. The chemical and electrical components of this force can be utilized, either in combination or separately, for carrier-mediated transport via substrate-proton symport.

While the importance of protons in carrier-mediated transport has been recognized for some time [4], only relatively few studies have shown the importance of Na^+ ions. Sodium-dependent growth and transport was first reported for marine bacteria [5,6], but later detected in several non-marine species where saturation seems to occur at much lower Na^+ concentrations [7–10]. Moreover,

National Research Council of Canada paper no. 23483.Abbreviations: Mes, 4-morpholineethanesulfonic acid; $TPMP^+$, triphenylmethylphosphonium; TPB^-, tetraphenylboron; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCS, 3,3′,4′,5-tetrachlorosalicylanilide.

Na^+-dependent transport systems can explain growth in alkaline media of *Escherichia coli* and alkalophilic bacilli (for review see Ref. 11). Generally, the mechanism for these effects is explained by the conversion of the protonmotive force to a sodiummotive force through Na^+/H^+ antiport, followed by substrate-sodium cotransport. It must be noted, however, that sodium gradients can be established in several ways in addition to Na^+/H^+ antiport (reviewed in Ref. 12 and 13). The sodiummotive force is composed of a membrane potential and a Na^+ chemical potential, defined as $\Delta\tilde{\mu}_{Na^+} = \Delta\psi - 60 \log([Na^+]_{out}/[Na^+]_{in})$ [13].

Vibrio costicola is a moderately halophilic bacterium which requires 1 M NaCl for optimal growth [14]. Carrier mediated transport of α-aminoisobutyric acid in these bacteria requires energy, depends on sodium ions, and is very salt tolerant; this tolerance (up to 4 M NaCl) depends on the NaCl concentration in which the cells are grown or to which they are exposed [15]. The pH optimum for growth extends from 6.5 well into the alkaline region (pH 9.0), where the pH gradient is reversed (inside acid). Both respiration-induced proton efflux and Na^+/H^+ antiport activities were identified at pH 7.5, where there is no measurable pH gradient. This suggested that antiport activity may depend on $\Delta\psi$ and may contribute to the formation of a sodiummotive force [12]. In this study we demonstrate the importance of $\Delta\psi$ and the sodiummotive force for the concentrative uptake of α-aminoisobutyric acid.

Materials and Methods

Growth and preparation of bacteria. V. costicola NRCC 37001 was grown aerobically as described previously [12]. Cultures were maintained in complex medium with 1.5% agar added, and stored at 4°C.

At the end of the exponential phase of growth (16–18 h, 1–1.5 mg dry wt. per ml) bacteria were washed twice with a solution of 1.0 M NaCl (or 1.0 M KCl when indicated) containing 8 mM KCl/0.4 mM $MgSO_4$/0.2 mM KH_2PO_4/50 mM Tris-HCl, final pH 7.2 (or as otherwise indicated). Suspensions were used immediately. When a series of experiments was carried out the suspension was stored at 0°C during the course of this series, in general for less than 2 h.

For salt solutions of different pH values, 50 mM Mes-KOH was used for pH values lower than 7.6 and 50 mM Tris-HCl for pH values higher than 7.2, in a 1.0 M NaCl solution with other salts as above. Solutions of NH_4Cl, LiCl, KCl and choline chloride contained 50 mM Tris-HCl (pH 8.8) and KCl, KH_2PO_4 and $MgSO_4$ as above. Bacterial dry weights were corrected for the weight of the salts as before [12].

Measurement of respiration. An oxygen electrode was placed in 5 ml of salt solution maintained at 30°C and stirred with a magnetic stirrer. Bacteria (1.0 mg dry wt.) and substrates (final concentration 20 mM) were added from Hamilton syringes. An immediate response (in O_2 utilization) was observed on addition of the bacteria. For experiments on oxidation of different electron donors (methanol, ethanol, propanol and butanol) the initial rate of respiration was measured for 2–3 min. In studying the effects of pH, inhibitors and different ions (KCl, NH_4Cl, LiCl and choline chloride), respiration was studied after incubation for 20 min at 30°C.

Since oxygen solubility changes with different dissolved salts, the apparatus was calibrated for each salt solution studied. The solutions were saturated with air, in the absence of bacteria; then the amount of dissolved oxygen was measured by iodometry, following the technique of Winkler [16]. For each series of experiments, the bacteria to be used were kept on ice with continuous aeration. Immediately after respiration was measured, the pH of each suspension of bacteria was measured using an Orion digital pH meter.

Measurement of intracellular volume. The intracellular volume using [^{14}C]urea and [^{3}H]raffinose was 1.81 μl/mg cell dry wt. [12].

Measurement of $\Delta\psi$. The magnitude of $\Delta\psi$ was determined at 30°C by measuring the accumulation of a lipophilic cation, triphenylmethylphosphonium ($TPMP^+$), in the presence of the anion, tetraphenylboron (TPB^-), as previously described [12].

Aminoisobutyric acid uptake. Bacteria (0.5 mg dry wt./ml) were incubated 20 min at 30°C in the salts medium indicated. α-Aminoisobutyric acid (200 μM, 0.5 μCi/μmol; New England Nuclear) was added and samples of 0.5 ml were filtered

through cellulose acetate filters (0.45 μm), and washed immediately with 5.0 ml of medium similar to which they had been incubated, without α-aminoisobutyric acid. Filters were placed in Aquasol (New England Nuclear) or ACS (Amersham) for counting.

Measurement of aminoisobutyric acid efflux. Bacteria (0.7–1.0 mg dry weight/ml) were loaded with α-amino[^{14}C]isobutyric acid by incubating for 30 min under conditions optimal for transport (pH 8.8 and 1 M NaCl). The cells were centrifuged (10 min, 7700 $\times$ *g*) at 4°C, and resuspended in the salt solution used for transport, but without α-aminoisobutyric acid. Aliquots of this bacterial suspension were then diluted into salt solutions (pH 8.8) containing 1 M KCl, LiCl, NH_4Cl or choline chloride, to a final concentration of 0.25–0.5 mg dry weight/ml. At the indicated times, 0.5 ml samples were filtered and treated as for the measurement of α-aminoisobutyric acid transport. Bacteria stored on ice in the presence of 1 M NaCl did not release significant amounts of α-amino[^{14}C]isobutyric acid during the course of the experiment.

Measurement of ATP. 1 ml of bacterial suspension (0.5 mg dry wt./ml) was added to 1.0 ml ice cold 12% $HClO_4$, incubated 20 min, and neutralized with a 0.72 M KOH/0.16 M $KHCO_3$ mixture [17]. ATP was reacted with luciferin-luciferase (Sigma) and monitored on the tritium channel of a scintillation counter [18].

Artificially imposed driving forces. Bacteria were washed in salt solution containing 1 M either of NaCl or of KCl, and final pH 7 or 9. Oxygen-free nitrogen (passed through a heated copper catalyst) was used to flush the cells (30 mg dry wt./ml) in 50 ml serum bottles for at least 30 min, at room temperature. The anaerobic suspensions of cells were then incubated at 30°C following closure with a butyl rubber stopper, and at intervals 100 μl aliquots were transferred with a Hamilton syringe to similar serum bottles containing the transport media under a nitrogen atmosphere (1 M NaCl, pH 9.0, α-amino[^{14}C]isobutyric acid 5 μM, 10 μCi/μmol). Samples of 0.5 ml were filtered as a function of time and washed with the salts medium used for transport.

Inhibitors. Ionophores, *N*,*N*′-dicyclohexylcarbodiimide (DCCD), and *N*-ethylmaleimide were prepared as methanolic solutions; appropriate controls were run to account for any effects of methanol alone. Treatments were for 20 min at 30°C.

Metabolism of aminoisobutyric acid. V. costicola was incubated with α-amino[^{14}C]isobutyric acid for 30 min at pH 8.8 as described for transport assays. The cells were extracted [12], and samples of the cell extract and medium subjected to thin layer chromatography on silica gel plates (250 μm, Analtech). Plates were developed with *n*-propanol: NH_4OH, 70 : 30 (v/v). The R_F value of authentic labeled compound was 0.46, as shown by autoradiography.

Results

Effect of external pH (pH_0) on aminoisobutyric acid uptake and respiration. Because the protonmotive force resulting from respiratory activity is the energy source for transport in many membrane systems, the effect of pH_0 on both α-aminoisobutyric acid uptake and respiration was studied. In Fig. 1 both activities are shown to be highest at alkaline pH_0 values, between 8.5 and 9; the same values at which $\Delta\psi$ is greatest (168 mV at pH 9.0)

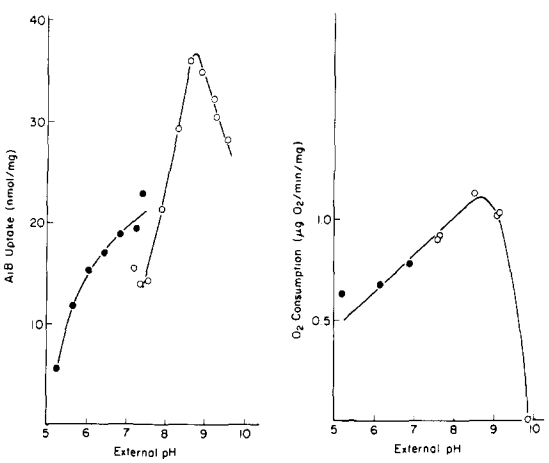

Fig. 1. Effect of external pH on α-aminoisobutyric acid uptake and the respiratory rate. Bacteria were suspended in a solution of 1 M NaCl, 8 mM KCl, 0.4 mM $MgSO_4$ and 0.2 mM KH_2PO_4 with 50 mM Tris-HCl (○) or 50 mM Mes-KOH (●). The bacteria were incubated at 30°C for 20 min before measuring α-aminoisobutyric acid uptake or respiratory rate. α-Aminoisobutyric acid uptakes were for 1.0 min, although a similar profile was obtained using 30 min uptake times. The pH values shown were measured at the conclusion of each test.

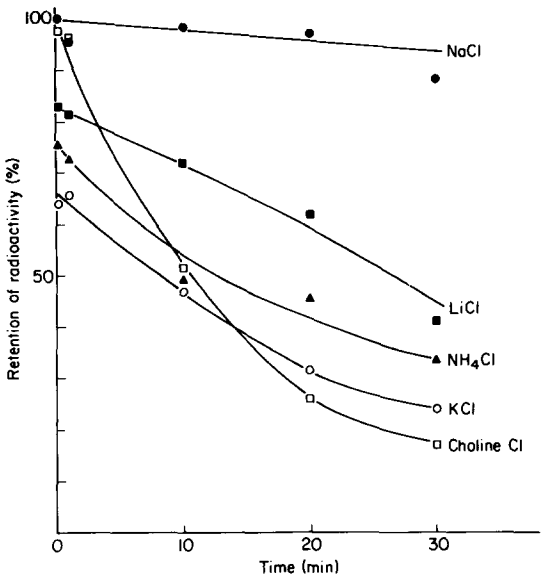

Fig. 2. Efflux of preloaded α-aminoisobutyric acid as influenced by the ionic environment. At time zero, cells of *V. costicola*, preloaded with α-amino[^{14}C]isobutyric acid, were placed in 1 M NaCl or 1 M of the various salts containing 20 mM NaCl. See Materials and Methods for details.

[12]. These results show a strong correlation between transport, respiration, and $\Delta\psi$. A sharp decline occurring in these activities between pH 9.8 and 9.9 (the lysis point of the cells) is illustrated in the figure by the respiration data. Although an energy source was not supplied for the experiment shown, both the endogenous and ethanol-stimulated respiration rates were maximal at the same alkaline pH_0 (results not shown).

No metabolism of α-aminoisobutyric acid was detected in cell extracts prepared after a 30 min uptake period.

Effect of ionic environment. V. costicola requires Na^+ both for growth and α-aminoisobutyric acid transport [14,15]. In addition, considerable efflux of α-aminoisobutyric acid from preloaded cells occurs when the cells are transferred to various salt solutions lacking NaCl (Fig. 2). The effect of the ionic environment on respiration, $\Delta\psi$ and ATP content of cells was next studied, in order to determine which factor(s) could be best correlated with ionic effects on transport. These experiments were carried out at the optimal pH_0 value (pH 8.5–9.0), determined above and by Hamaide et al. [12].

As previously shown [15], *V. costicola* can respire in 1.0 M KCl in the absence of Na^+; the cells also respire in 1 M LiCl, NH_4Cl or choline chloride (Table I). In contrast to the case with the other ions, a large proportion of the cells lyse when suspended in 1 M choline chloride (without Na^+) invalidating its use as an osmotic stabilizing agent. Additional oxygen uptake occurs in the presence of ethanol in all salts except choline

TABLE I

EFFECT OF VARIOUS IONS ON α-AMINOISOBUTYRIC ACID TRANSPORT, $\Delta\psi$, O_2 CONSUMPTION, AND INTRACELLULAR ATP CONCENTRATION

All assays performed at pH 8.5–8.8. N.d., not done.

Ion (1 M)	α-aminoisobutyric acid transport [a] (nmol/mg cells)				$\Delta\psi$ Endogenous		O_2 Consumption (μg O_2/min per mg cells)		ATP Concentration [b] (μg/mg cells)	
	Endogenous		Ethanol [c]		$\frac{[TPMP]_{in}}{[TPMP]_{out}}$	(mV)	Endogenous	Ethanol [c]	Endogenous	Ethanol
	1 min	30 min	1 min	30 min						
NaCl	29	163	52	227	420	157	1.0	2.4	4.27	6.51
KCl	2	8	3	7	19	76	1.3	1.9	0.88	3.06
LiCl	0	0	N.d.	N.d.	38	95	0.25	1.2	N.d.	N.d.
NH_4Cl	0	3	N.d.	N.d.	27	78	0.57	2.1	N.d.	N.d.
Choline Cl	0	0	N.d.	N.d.	20	86	0.56	0.56	N.d.	N.d.

[a] Transport was measured after 1 min and 30 min uptake periods.
[b] ATP was extracted from cells which had taken up α-aminoisobutyric acid for 30 min.
[c] Ethanol (20 mM) was added to the cell suspension about 10 s prior to initiating the assays.

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF KCl AND NaCl ON $\Delta\psi$ AND α-AMINOISOBUTYRIC ACID TRANSPORT

Bacteria were washed twice in salt solution containing either 1 M NaCl or 1 M KCl (pH 7.2) and incubated 20 min in salt solutions containing various concentrations of NaCl and KCl (pH 8.8). Standard errors are shown for two independent experiments.

Salt solution		Washing solution					
KCl (M)	NaCl (M)	KCl			NaCl		
		$\Delta\psi$ (mV)	α-Aminoisobutyric acid transport (nmol/mg)		$\Delta\psi$ (mV)	α-Aminoisobutyric acid transport (nmol/mg)	
			1 min	30 min		1 min	30 min
0	0	37±53	1±1	1± 1	39±56	0	0
0.5	0	37±52	0	0	76±18	0	0
1	0	51±73	0	0	0	1±1	8± 2
0	0.5	148± 3	30±3	144±29	152±10	28±4	124±11
0.5	0.5	138± 5	16±3	104±21	139± 4	17±1	90±10
1	0.5	121± 4	13±1	75±19	132± 8	13±1	74± 6
0	1	154± 1	33±4	174±29	157± 1	31±3	174±15
0.5	1	123±15	14±2	79±12	148± 1	15±2	92± 1
1	1	123±19	7±3	44± 4	142± 7	7±1	51± 3

chloride. Furthermore, ATP is produced by the cells respiring in the presence of ethanol and KCl, though the concentration of ATP maintained in cells carrying out endogenous respiration in KCl is quite low. In contrast, though ethanol strongly stimulates α-aminoisobutyric acid transport in cells suspended in 1 M NaCl, no such stimulation is observed in cells suspended in 1 M KCl; in the latter cells, no transport at all is observed.

$\Delta\psi$ is greatly lowered in KCl, LiCl, NH_4Cl and choline chloride in the absence of Na^+ (Table I). These low values for $\Delta\psi$ represent small distributions of $TPMP^+$ (in/out) relative to that found when Na^+ is present, and may therefore be subject to overestimation resulting from non-specific binding of $TPMP^+$ [12]. This effect on $\Delta\psi$ indicates either that Na^+ is specifically required in the formation or maintenance of an appreciable $\Delta\psi$, or that ions other than Na^+ dissipate the potential. To distinguish between these possibilities, cells washed in 1 M KCl were tested to see if $\Delta\psi$ (and α-aminoisobutyric acid transport) recovered upon NaCl addition (Table II). By comparing to the NaCl control, it is evident that $\Delta\psi$ recovered fully

TABLE III

EFFECT OF SOME ELECTRON DONORS ON THE RESPIRATORY RATE, α-AMINOISOBUTYRIC ACID TRANSPORT, $\Delta\psi$ AND ATP CONTENT

All measurements were initiated about 10 s following the addition of the alcohols. ATP was extracted from cells incubated for 30 min with α-aminoisobutyric acid.

Energy supply	Respiratory rate ($\mu g\ O_2$/min per mg)	α-Aminoisobutyric acid transport (nmol/mg)		$\Delta\psi$ (mV)	ATP content (μg/mg)
		1 min	30 min		
Endogenous	2.20	29	173	147	5.34
Methanol (20 mM)	2.15	26	168	152	5.34
Ethanol (20 mM)	3.14	63	325	151	9.57
Propanol (20 mM)	3.21	66	313	148	9.07
Butanol (20 mM)	2.61	42	227	146	8.95

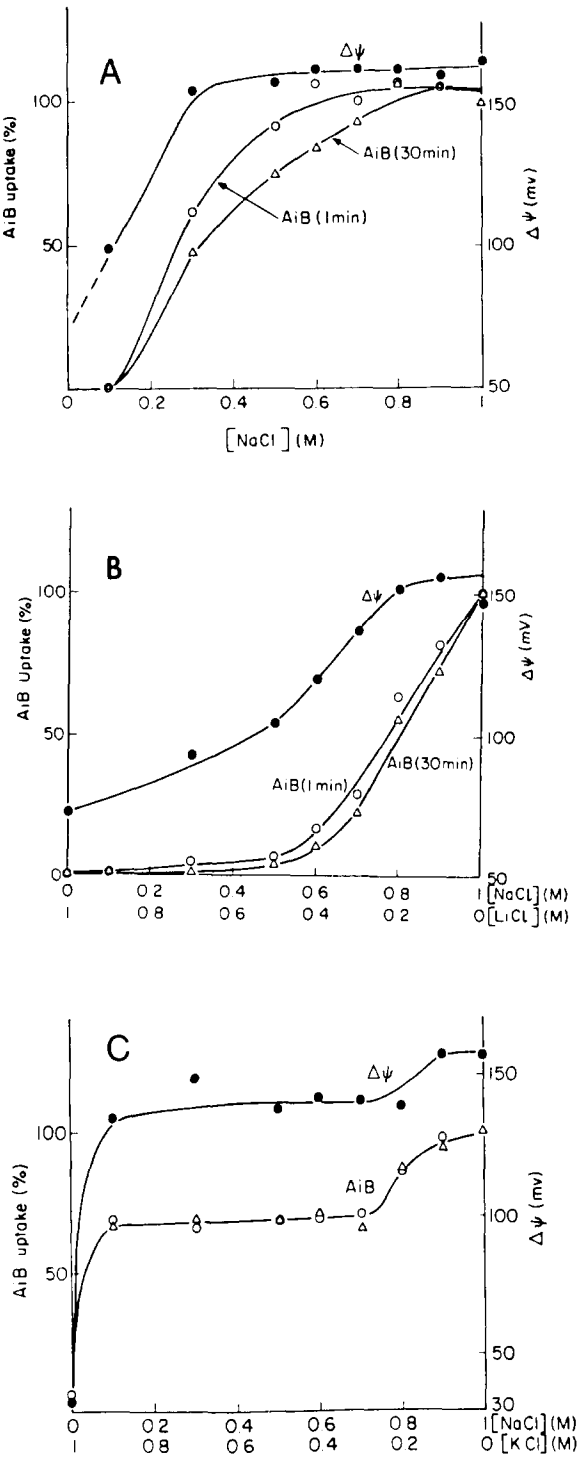

Fig. 3. Influence of the ionic environment on α-aminoisobutyric acid uptake and Δψ. Bacteria were incubated for 20 min (pH 8.8) with the indicated concentrations of NaCl varied from 0 to 1.0 M (panel A), or with an osmolarity of 1.0 maintained, using LiCl (panel B) or KCl (panel C). Values corresponding to 100% uptake for 1.0 and 30 min in nmol/mg dry wt. are 30 and 170 (panel A), 24 and 137 (panel B), and 30 and 160 (panel C), respectively.

upon the readdition of NaCl to the cells, and that the continued presence of KCl at high concentration (either 0.5 or 1.0 M) was only moderately inhibitory to Δψ. These studies show that Na^+ is necessary for the formation and maintenance of Δψ. A positive correlation was seen between Δψ and α-aminoisobutyric acid uptake throughout this study on ion effects.

To investigate further the relationship between Δψ and α-aminoisobutyric acid uptake, bacteria were incubated in different concentrations of NaCl (Fig. 3). As the NaCl concentration was lowered below 0.3 M without any compensation for lowered osmolarity (panel A), both α-aminoisobutyric acid uptake and Δψ markedly decreased. With *V. costicola* lysis begins at 0.13 M NaCl [15]. Since at 0.3 M NaCl Δψ was near its maximum value and α-aminoisobutyric acid uptake had declined to about 50% of maximum, this provides evidence for a role(s) of Na^+ for α-aminoisobutyric acid transport additional to that for Δψ (see also Table I). When osmolarity of 1.0 was maintained with LiCl (panel B), marked parallel inhibitions of both Δψ and α-aminoisobutyric acid uptake were evident. By comparing with panel A, it is evident that LiCl has an inhibitory effect. Less inhibition was obtained by using KCl to maintain osmolarity of 1.0, since no changes in Δψ or α-aminoisobutyric acid uptake occurred in the range 0.1–0.9 M KCl. In all cases the correlation between α-aminoisobutyric acid uptake and Δψ was striking.

Electron donors. To test the effects of electron donors on α-aminoisobutyric acid transport, it was best to use substrates which should penetrate the cells independent of Na^+ or energy. Alcohols have been used successfully for this purpose in *Alteromonas haloplanktis* [19,20]. The effects of alcohols were measured in cell suspensions containing 1 M NaCl, at pH_0 of 8.8 where no positive contribution to α-aminoisobutyric acid uptake is expected from ΔpH (Table III). The alcohols which immediately stimulated respiration (ethanol, propanol and butanol) also increased ATP concentra-

tions and α-aminoisobutyric acid transport, but produced no change in $\Delta\psi$. Incubation of the cells with ethanol for either a few seconds or for 20 min before adding [^{3}H]TPMP$^+$ produced no change in $\Delta\psi$. Methanol produced no changes in any of the parameters tested. These results suggest that the energy generated by respiration, during oxidation of electron donors, is not necessarily conserved as a measurable form of $\Delta\psi$ but may, rather, be used immediately for functions such as ATP synthesis or carrier mediated transport of α-aminoisobutyric acid.

Inhibitors. DCCD is an inhibitor of ATPase activity in mitochondria and numerous bacteria including *V. costicola* [21,22].DCCD, when less than 40 μM, stimulated α-aminoisobutyric acid transport and respiration. $\Delta\psi$ remained unchanged and the ATP content of the cells decreased greatly, presumably through inhibition of the ATPase (Fig. 4). At higher concentrations the specificity for the ATPase was lost, since DCCD inhibited respiration and α-aminoisobutyric acid transport, and dissipated $\Delta\psi$. These results suggest that ATP is not a major energy source for α-aminoisobutyric acid transport.

N-ethylmaleimide, which reacts with sulfhydryl groups, and KCN have pronounced effects on the respiratory chain of *V. costicola* and on $\Delta\psi$ (Table IV). In the presence of these inhibitors, no α-aminoisobutyric acid transport takes place.

A decline in $\Delta\psi$ occurs with the protonophores carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3,3′,4′,5-tetrachlorosalicylanilide (TCS). Both CCCP and TCS act as protonophores in *V. costicola* [12] but may inhibit α-aminoisobutyric acid transport by more than one mechanism (see below). Moreover, the protonophores behave

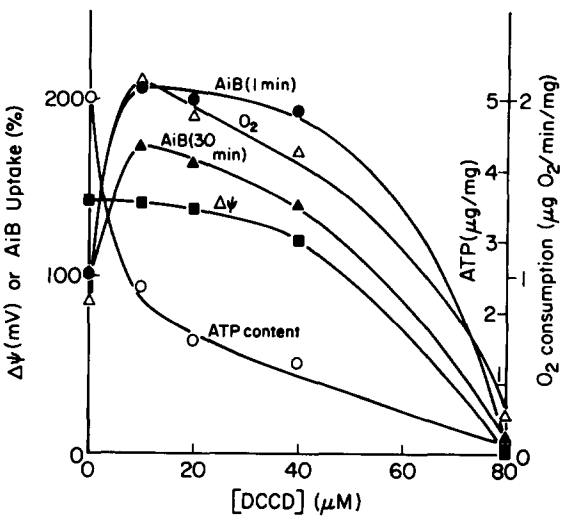

Fig. 4. Inhibitory effects of DCCD on α-aminoisobutyric acid uptake, ATP content, respiration rate, and $\Delta\psi$. Bacteria were incubated for 20 min, at 30°C, with DCCD (1 M NaCl, pH 8.8) prior to performing the various assays. In the case of ATP extractions, these were carried out following the 30 min uptake of α-aminoisobutyric acid. Endogenous activities were measured. Values corresponding to 100% uptake in nmol/mg dry wt. are 29 and 151, for 1.0 and 30 min, respectively.

TABLE IV

EFFECT OF INHIBITORS ON THE RESPIRATORY RATE, $\Delta\psi$ AND α-AMINOISOBUTYRIC ACID TRANSPORT

Treatment	Activity (% of uninhibited) [a]		
	Respiration rate	$\Delta\psi$	α-Aminoisobutyric acid transport (1.0 min)
N-ethylmaleimide (10 mM)	18	17	0
KCN (10 mM)	0	8	8
TCS (20 μM)	70	28	1
CCCP (20 μM)	265	32	2
Valinomycin (20 μM)+KCl (0.5 M)	not done	49	53
Monensin (20 μM)	262	39	24
Nigericin (20 μM)	186	3	2
Gramicidin (10 μg/ml)	92	108	61

[a] 100% of activity = 0.58 μg O_2/min per mg for the endogenous respiration; 156 mV for $\Delta\psi$; 27.5 nmol/mg for α-aminoisobutyric acid transport.

differently, since CCCP stimulates and TCS partially inhibits respiration. A complete dissipation of $\Delta\psi$ by valinomycin (plus KCl) does not occur, as expected from previous results showing that valinomycin is unable to catalyze any appreciable uptake of $^{86}Rb^+$ in *V. costicola* [12].

Nigericin often elicits an electroneutral exchange of H^+ and K^+, monensin an electroneutral exchange of H^+ and Na^+, and gramicidin which acts as a rather nonspecific conductor of H^+, Na^+ and K^+ collapses $\Delta\psi$ [23]. In spite of a large increase in respiration rate, treatment with nigericin and monensin resulted in the partial collapse of $\Delta\psi$ and α-aminoisobutyric acid transport (Table IV). A role for monensin in catalyzing the influx of protons with collapse of $\Delta\psi$ is consistent with previous results showing that monensin mimics the effects of protonophores by abolishing net proton efflux in response to an oxygen pulse [12]. Hence, monensin and nigericin behave much like protonophores in this system. Gramicidin exerted little effect on either respiration or $\Delta\psi$, but produced a small but significant decline in α-aminoisobutyric acid transport. Although we do not know why gramicidin is ineffective, these results on $\Delta\psi$ correlate to an absence of growth inhibition by 20 μM gramicidin added to a defined medium, pH 8.5 (not shown).

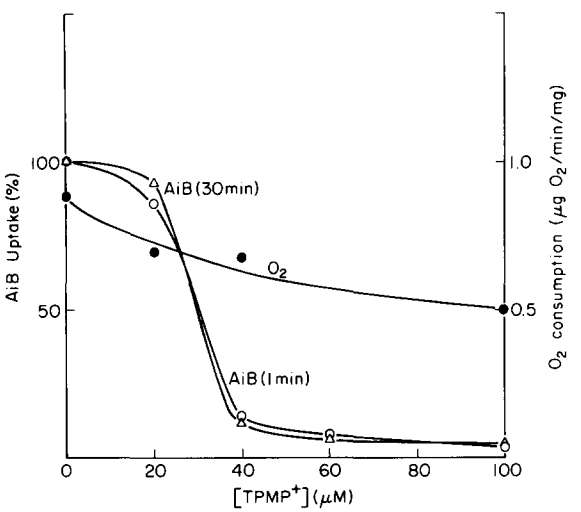

Fig. 5. Influence of unlabeled-$TPMP^+$, in the presence of 2 μM TPB^-, on α-aminoisobutyric acid uptake and respiration. Incubation with $TPMP^+$ was for 20 min at 30°C before determining α-aminoisobutyric acid uptake and respiration rate. Measurements were made in the absence of an added energy source with cells exposed to 1 M NaCl, pH 8.8. Similar results for respiration (not shown) were obtained using 20 mM ethanol as an energy source. Transport rates for control cells were as shown in Fig. 4.

The effects of the lipophilic cation, $TPMP^+$ (in the presence of TPB^-, 2 μM) were measured on both α-aminoisobutyric acid transport and respiration (Fig. 5). Concentrations higher than 40 μM completely inhibited α-aminoisobutyric acid transport. In contrast, the capacity of the bacteria to respire diminished only slightly with increasing $TPMP^+$ concentrations. The lipophilic cation is taken up by the cells, and should abolish $\Delta\psi$ according to the concentration of the cation added [24]. Hence, these results support the conclusion that $\Delta\psi$ is required in α-aminoisobutyric acid transport at alkaline pH_0.

Aminoisobutyric acid uptake in response to artificially created energy sources. Attempts were unsuccessful to demonstrate α-aminoisobutyric acid uptake into anaerobic cell suspensions when the conditions described by Tsuchiya et al. [8,9,25] were used to establish artificial energy sources. This included reaction of $\Delta\psi$ (in the presence of Na^+) by suddenly shifting the pH of the cell suspension (pH 7–9) in the presence of the protonophores CCCP or TCS. However, considerable concentration gradients of α-aminoisobutyric acid were achieved in response to a pH shift, providing protonophores were omitted (Fig. 6). These protonophores exert more potent effects on α-aminoisobutyric acid transport than on $\Delta\psi$ (Table IV). Together, these results suggest a site(s) of inhibition additional to the energy supply. Data have been presented for a direct interaction of these protonophores with certain amino acid or sugar translocators in other microorganisms (see Ref. 26). In the absence of protonophores, $\Delta\psi$ may form as a result of proton efflux through the anaerobic membrane where initially the ΔpH is acidic inside [12]. This would require a membrane somewhat permeable to protons under the test conditions. In support of formation of $\Delta\psi$ in response to a pH shift, high concentrations of $TPMP^+$ partially prevented the uptake of α-aminoisobutyric acid (as in Fig. 5) and only slight uptake occurred in the absence of a pH shift. Also, direct measurements consistently showed the formation of $\Delta\psi$ (inside negative) in response to the

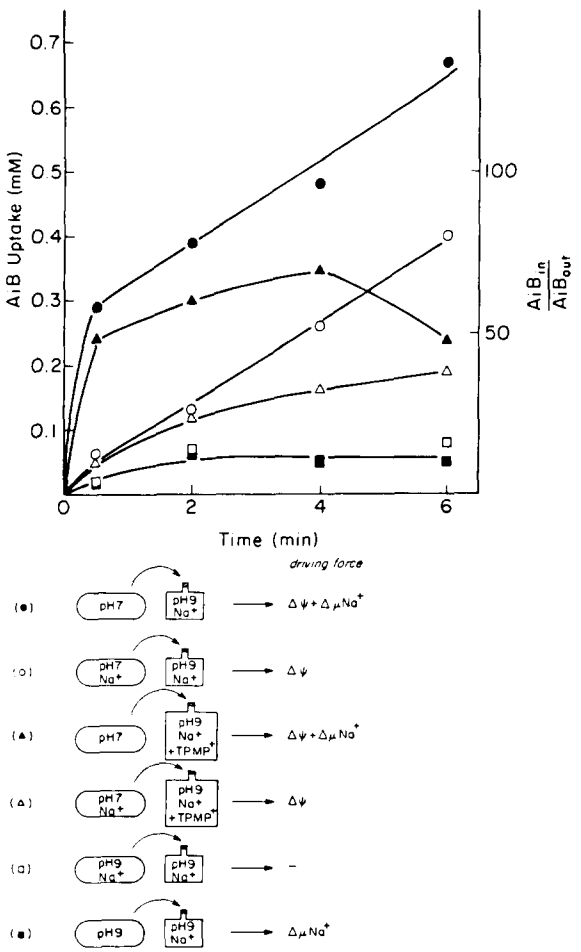

Fig. 6. α-Aminoisobutyric acid uptake in response to artificially imposed driving forces. The details described in Materials and Methods were followed, as illustrated in the figure. Unlabeled $TPMP^+$ was used, where indicated, at 100 μM in the presence of 2 μM TPB^-. Anaerobic precautions were taken throughout. *V. costicola* was initially in a 1 M KCl solution under an N_2 atmosphere, then a rapid pH shift and/or a rapid addition of 1 M NaCl followed. An intracellular concentration of 1 mM α-aminoisobutyric acid corresponds to 1.8 nmol/mg dry wt.

pH shift. These measurements varied quantitatively among experiments for reasons unknown. The kinetics of α-aminoisobutyric acid uptake are less transient in *V. costicola* than in other systems employing a protonophore [8,25].

To establish an Na^+ chemical potential, a concentrated cell suspension in 1 M KCl was rapidly shifted to a solution containing NaCl (with or without a pH shift to create $\Delta\psi$). No uptake occurred in response to the Na^+ gradient alone, but uptake above that observed for a pH shift alone was observed in response to the combined driving forces. The kinetics of α-aminoisobutyric acid uptake show a slow constant rate in response to the pH shift and a fast uptake attributable to the additional effect of the Na^+ chemical potential. Although $\Delta\psi$ was obligatory for uptake, for maximum rates the Na^+ chemical potential was also necessary.

Discussion

Sodium ions may play several roles in the functions of the cytoplasmic membrane. Depending on the bacterial species, the ion may be required to prevent lysis and to stabilize the cytoplasmic membrane, to activate enzymes, to establish a sodium motive force or to change the K_m of the carrier for the substrate. *V. costicola* requires 1 M NaCl for optimal growth, and lysis occurs in water [15,27]. Lysis may be prevented by salts other than NaCl including LiCl, KCl or NH_4Cl. Since the bacteria respire in these salt solutions, a requirement for Na^+ at the level of the respiratory enzymes is unlikely ([12]; Table I). ATP is synthesized in the absence of added Na^+ as well (Table I), suggesting no requirement of Na^+ for ATPase activity. However, at least three functions depend specifically on the presence of the Na^+ ion. These include the uptake and retention of α-aminoisobutyric acid (this study), the regulation of internal pH in alkaline media through Na^+/H^+ antiport activity [12] and the formation and maintenance of $\Delta\psi$ (this study). Li^+ ions cannot substitute well for Na^+ in these functions and are even inhibitory to α-aminoisobutyric acid transport and $\Delta\psi$ (Fig. 3). All of the mentioned Na^+-dependent functions are recovered when NaCl is added to *V. costicola* stored in a 1 M KCl salt solution, showing that membrane integrity was conserved in the absence of NaCl. A role for Na^+ in lowering the apparent K_m for α-aminoisobutyric acid transport approx. 10-fold has been found (Hamaide, F., Sprott, G.D. and Kushner, D.J., unpublished data).

Transport of the non-metabolized amino acid (α-aminoisobutyric acid) in *V. costicola* is carrier mediated. Competition experiments (not shown) using 20 μM each of labeled α-aminoisobutyric acid and the competing amino acid revealed in-

hibitions of 74, 48 and 18%, respectively, for glycine, L-alanine, and L-methionine; branched chain amino acids and L-valine were not inhibitory. Typical transport kinetics were obtained with an apparent K_m of $2.0 \cdot 10^{-5}$ M and a maximum velocity of 41 nmol/min per mg dry wt. (Hamaide, F., Sprott, G.D. and Kushner, D.J., unpublished data). In these respects, α-aminoisobutyric acid transport resembles that found in the marine bacterium *Alteromonas haloplanktis* [28].

α-Aminoisobutyric acid transport in *V. costicola* is energy dependent, showing a close relationship to the respiratory rate (Fig. 1, Table III). Several lines of evidence support the conclusion that the transport system exhibits an obligatory requirement for an appreciable $\Delta\psi$. Variations in the magnitude of $\Delta\psi$, as measured by $TPMP^+$ distributions, result in corresponding changes in α-aminoisobutyric acid transport; both $\Delta\psi$ and transport exhibit similar pH profiles with optima of 8.5–9.0, and both experience corresponding changes in response to the ionic environment and to the presence of inhibitors.

The involvement of an energy source additional to $\Delta\psi$ is suggested by enhanced rates of α-aminoisobutyric acid transport upon addition of alcohols, which stimulate respiration but do not increase $\Delta\psi$. Similarly, appropriate concentrations of DCCD enhanced both respiration rate and α-aminoisobutyric acid transport without changing the magnitude of $\Delta\psi$. Although changes in the respiratory rate and α-aminoisobutyric acid transport, induced by adding various alcohols, revealed a concomitant change in ATP content (Table III), no such correlation was seen during inhibitions by DCCD (Fig. 4). Low concentrations of DCCD caused ATP to decline while respiration and α-aminoisobutyric acid uptake were stimulated. This lack in correlation between the ATP content and transport suggests that ATP is not the additional energy supply for α-aminoisobutyric acid transport described above.

The reason for the stimulation of respiration by low concentrations of DCCD is not fully understood. A perturbation in the energy charge of the adenylate pool may have activated pathways [29] to produce more reducing equivalents. Since respiration in these endogenous cells is less than maximal, an enhanced rate of O_2 uptake could be expected.

An increase in the respiration rate on addition of alcohols can also be explained by increased rates of NADH formation (and oxidation). The lack of a concomitant change in $\Delta\psi$ suggests that a maximum $\Delta\psi$ is already maintained with the endogenous energy supply, such that additional protons once expelled leak back inside (partially through the ATPase complex to explain the observed rise in ATP content) or that the stoichiometry of charge movement becomes electroneutral at high values of $\Delta\psi$. This would be the case if the stoichiometry of respiratory-induced H^+ efflux to Na^+/H^+ movement through the antiporter were 2 : 1 : 3. Conditions exist where the net flux of H^+ is inwardly directed, since an alkalinization of the medium (containing NaCl) occurs at pH_0 8.8 when anaerobic cells of *V. costicola* receive an O_2-pulse (data not shown). However, this would not be the stoichiometry expected at lower values of $\Delta\psi$, since respiration coupled with antiport activity may be the normal means to form $\Delta\psi$ at alkaline pH_0 [12]. Similar explanations may apply to the DCCD results where $\Delta\psi$ does not increase at increased rates of respiration.

Effects of increased respiration may be explained if the Na^+ chemical potential becomes larger under these conditions. The enhanced respiratory rates and increased rates of proton efflux are expected to increase Na^+/H^+ antiport activity, as required to maintain a relatively constant cytoplasmic pH, resulting in an increased Na^+ gradient. Previously, we have shown that respiratory activity in cells suspended in an Na^+-free solution results in proton efflux at pH 7.5 [12] and also at pH 8.8 (unpublished data) where both respiration and transport are maximal. Results with artificially established driving forces indicate that this additional energy source is the Na^+ chemical potential. Little α-aminoisobutyric acid transport occurred in response to the Na^+ chemical potential alone, but the transport observed in response to a pH shift (creating $\Delta\psi$) was enhanced by the combination of both energy sources (Fig. 6). Measurements of α-aminoisobutyric acid transport in response to artificial driving forces led to the same conclusions for the marine bacterium, *Alteromonas haloplanktis* [30]. Although direct evidence for Na^+/α-aminoisobutyric acid symport is difficult to obtain [30,31], α-aminoisobutyric acid

transport in *V. costicola* by such a mechanism would explain the influence of the chemical gradient of Na^+ ions.

Marked differences in energy coupling are evident between *V. costicola and V. alginolyticus.* Both organisms require NaCl, with optimal growth of *V. costicola* in 1 M NaCl [14], and *V. alginolyticus* in 0.5 M [32]. When Na^+-loaded, both organisms respire best at alkaline external pH and generate a maximal $\Delta\psi$, inside negative [12,33]. However, the mechanisms of forming $\Delta\psi$ at alkaline pH appear to be different. A respiration-dependent primary Na^+ pump is operative at alkaline external pH in *V. alginolyticus,* which generates a $\Delta\psi$ insensitive to protonophores [34]. Consequently, a Na^+-requirement for respiration can be demonstrated above pH 8.0 [34]. In *V. costicola* respiration causes proton extrusion even at pH 8.5–9.0 and $\Delta\psi$ is collapsed by protonophores (Ref. 12, and present study). Respiration at external pH 8.8 showed no significant requirement for Na^+, while certain ions such as Li^+ were inhibitory (Table I). Finally, *V. alginolyticus* formed $\Delta\psi$ in the presence of only 50 mM Na^+ [33], while in this study we demonstrated in *V. costicola* an absolute requirement for much higher Na^+ concentrations to generate and maintain $\Delta\psi$.

Acknowledgements

This work was supported, in part, by a grant from the Natural Sciences and Engineering Research Council of Canada to D.J.K., and by a scholarship from the World University Service of Canada to F.H.

References

1 Harold, F.M. (1977) Curr. Top. Bioenerg. 6, 83–149
2 Rosen, B.P. and Kashket, E.R. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 559–620, Marcel Dekker, New York
3 West, I.C. (1980) Biochim. Biophys. Acta 604, 91–126
4 West, I.C. (1970) Biochem. Biophys. Res. Commun. 41, 655–661
5 Drapeau, G.R., Matula, T.I. and MacLeod, R.A. (1966) J. Bacteriol. 92, 63–71
6 Wong, P.T.S., Thompson, J. and MacLeod, R.A. (1969) J. Biol. Chem. 244, 1016–1025
7 Frank, L. and Hopkins, I. (1969) J. Bacteriol. 100, 329–336
8 Tsuchiya, T., Hasan, S.M. and Raven, J. (1977) J. Bacteriol. 131, 848–853
9 Tsuchiya, T., Raven, J. and Wilson, T.H. (1977) Biochem. Biophys. Res. Commun. 76, 26–31
10 Stock, J. and Roseman, S. (1971) Biochem. Biophys. Res. Commun. 44, 132–138
11 Padan, E., Zilberstein, D. and Schuldiner, S. (1981) Biochim. Biophys. Acta 650, 151–166
12 Hamaide, F., Kushner, D.J. and Sprott, G.D. (1983) J. Bacteriol. 156, 537–544
13 Lanyi, J.K. (1979) Biochim. Biophys. Acta 559, 377–397
14 Forsyth, M.P. and Kushner, D.J. (1970) Can. J. Microbiol. 16, 253–361
15 Kushner, D.J., Hamaide, F. and MacLeod, R.A. (1983) J. Bacteriol. 153, 1163–1171
16 Winkler, L.W. (1888) Berl. Dtsch. Chem. Ges. 21, 2843–2854
17 Bagnara, A.S. and Finch, L.R. (1972) Anal. Biochem. 45, 24–34
18 Strehler, B.L. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U. and Gawehn, K., eds.), Vol. 4, pp. 2115–2126, Academic Press, New York
19 Sprott, G.D. and MacLeod, R.A. (1974) J. Bacteriol. 117, 1043–1054
20 Thompson, J. and MacLeod, R.A. (1974) J. Bacteriol. 117, 1055–1064
21 Higa, A.I. and Cazzulo, J.J. (1978) FEMS Lett. 3, 157–160
22 Bengis-Garber, C. and Kushner, D.J. (1981) J. Bacteriol. 146, 24–32
23 Bakker, E.P. (1979) in Antibiotics, Vol. V, part 1, Mechanism of Action of Antibacterial Agents (Hahn, F.E., ed.), pp. 67–97, Springer-Verlag, Berlin
24 Michel, H. and Oesterhelt, D. (1976) FEBS Lett. 65, 175–178
25 Hasan, S.M. and Tsuchiya, T. (1977) Biochem. Biophys. Res. Commun. 78, 122–128
26 Van den Broek, P.J.A. and Van Steveninck, J. (1983) Biochim. Biophys. Acta 722, 102–106
27 Christian, J.H.B. and Ingram, M. (1959) J. Gen. Microbiol. 20, 32–42
28 Fein, J.E. and MacLeod, R.A. (1975) J. Bacteriol. 124, 1177–1190
29 Atkinson, D.E. (1968) Biochemistry 7, 4030–4034
30 Niven, D.F. and MacLeod, R.A. (1980) J. Bacteriol. 142, 603–607
31 Tokuda, H., Sugasawa, M. and Unemoto, T. (1982) J. Biol. Chem. 257, 788–794
32 Unemoto, T., Hayashi, M. and Terao, K. (1977) Biochim. Biophys. Acta 500, 425–431
33 Tokuda, H., Nakamura, T. and Unemoto, T. (1981) Biochemistry 20, 4198–4203
34 Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014