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TRANSPORT OF α-AMINOISOBUTYRATE BY CELLS AND MEMBRANE VESICLES OF *PSEUDOMONAS FLUORESCENS*

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Summary

The transport of α-aminoisobutyrate into *Pseudomonas fluorescens* NCIB 8865 and membrane vesicles prepared from this organism has been studied. Uptake by cells was mediated by two active transport systems with different apparent K_m values, while transport into membrane vesicles was mediated by a single component. The effect of inhibitors on the energy-coupling mechanism for α-aminoisobutyrate transport in these systems suggests that a membrane potential may play a significant role in supporting α-aminoisobutyrate transport. The magnitude of the membrane potential in the vesicle system, and the sensitivity of its generation to inhibitors, has been measured using ^{137}Cs in the presence of valinomycin. Direct attempts to demonstrate a proton-symport mechanism for α-aminoisobutyrate transport were negative.

Introduction

During the last decade membrane vesicles have been used increasingly in the investigation of the mechanism of energy coupling to active transport in bacterial systems [1—3]. In parallel with these studies has been the development of the chemiosmotic coupling hypothesis suggesting that in many membrane-bound systems energy is conserved in the form of a proton-motive force (electrochemical gradient of H^+). Such a force is suggested to play a key role in the energy-linked reactions associated with the membrane including driving active transport systems [4—6]. With the recent reports from Kaback's laboratory [7—9] these studies have converged to confirm this role. Thus, membrane vesicles have been shown to generate a proton-motive force of

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DDACl, dibenzyldimethylammonium chloride; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, *N,N,N'N'*,-tetramethyl-*p*-phenylenediamine dihydrochloride; TPMPBr, methyltriphenylphosphonium bromide.

sufficient magnitude to account for substrate accumulation ratios [10] and a good correlation between the ability of various electron donors to drive transport and generate a proton-motive force has been demonstrated [7].

Studies on the mechanism of energy coupling to nutrient transport in *Pseudomonads* have been relatively limited apart from an extensive study of α-aminoisobutyrate transport in a marine *Pseudomonad* B-16, in which MacLeod and his co-workers [11,12] have demonstrated that transport was dependent on Na^+, but have eliminated a role for Na^+ gradients in the energy-coupling mechanism [13]. It has been reported [14] that maltose transport in an α-glucosidase-negative strain of *Pseudomonas fluorescens* W required an energized-membrane state, and that membrane vesicles prepared from *P. fluorescens* W could utilize phenazine methosulphate-ascorbate to support maltose transport. Similarly, methionine transport in membrane vesicles prepared from *P. fluorescens* UK1 was stimulated by both natural electron donors such as succinate and 2-oxoglutarate and artificial electron donor systems such as phenazine methosulphate-ascorbate and FAD-malate [15].

The work presented in this paper extends these studies, being concerned with the mechanism of energy coupling to α-aminoisobutyrate transport in *P. fluorescens* NCIB 8865. Transport has been investigated in the parent organism and membrane vesicles prepared from it; the magnitude of the membrane potential has been estimated, and a role for this potential as a driving force for α-aminoisobutyrate transport has been investigated.

Materials and Methods

Organism and growth of bacteria. The organism used was *P. fluorescens* NCIB 8865 and was maintained on nutrient agar slants. The bacteria were grown at 30°C in 250-ml batches of nutrient broth (Difco Laboratories, 8 g/l) containing glucose (8 g/l) in 1-l Erlenmeyer flasks on a gyrotary shaker (New Brunswick Scientific Co.). Inocula (0.1%) were taken from 10-ml batch cultures on nutrient broth (8 g/l) subcultured daily, and after 16 h growth the bacteria were harvested, in the stationary phase, at a density of approx. 1.5 mg cell dry wt./ml.

Preparation of membrane vesicles. Bacteria were harvested by centrifuging at 5000 × *g* for 10 min at 4°C, washed once with 10 mM Tris · HCl, pH 7.1, resuspended in 100 ml of 75 mM Tris · HCl, pH 8.0, and added to 100 ml of 50% (w/v) sucrose and 50 ml of 75 mM K_2EDTA. Lysozyme was added to a final concentration of 0.5 mg/ml and the suspension was incubated with stirring at room temperature for 45 min. The spheroplasts formed were harvested by centrifuging at 12 000 × *g* for 15 min at 4°C, and were resuspended, using a Teflon and glass homogenizer operated manually, in the smallest possible volume of 0.1 M KH_2PO_4, pH 6.6, containing 20% (w/v) sucrose and 20 mM $MgSO_4$. The spheroplasts were lysed by dilution into 250 ml of 10 mM KH_2PO_4, pH 6.6, prewarmed to 25°C, and stirred continuously during lysis. A few crystals of DNAase were added, followed by $MgSO_4$ to a final concentration of 20 mM and stirring at room temperature was continued until the suspension was no longer viscous. The lysate was centrifuged for two periods of 10 min at 7700 × *g* and 4°C and the pellets were

discarded. The supernatant fluid was then centrifuged at 48 200 × *g* for 30 min at 4°C to sediment the vesicles. The harvested membranes were washed by gentle resuspension in 0.1 M KH_2PO_4, pH 6.6, and were recentrifuged before being resuspended in an identical buffer and stored on ice. In order to study the effect of the ionic composition of the medium on α-aminoisobutyrate transport, vesicles were also prepared as previously described replacing the 10 mM and 100 mM sodium potassium phosphate buffers with 10 mM and 100 mM HEPES/Tris, pH 7.1.

Transport studies. Bacteria were harvested by centrifuging at 5000 × *g* for 10 min at 4°C, washed twice with either 0.067 M KH_2PO_4, pH 7.1, containing 0.1% (w/v) $MgSO_4$, or 0.05 M HEPES/Tris, pH 7.1, and were stored on ice. Transport was assayed at 25°C using the methods described previously [16]. The wash fluids used comprised the appropriate buffers described above containing 2% (w/v) NaCl. Uptake rates were measured in duplicate from four 0.5-ml samples taken during the first 2 min and are expressed as μmol/min per g dry wt.

Transport studies using membrane vesicles. All assays were performed on freshly prepared vesicles, suspensions of which were stored on ice at a density between 2 and 4 mg protein/ml for vesicles in phosphate buffer and 5 and 10 mg/ml for vesicles in HEPES/Tris. When glucose was used to support transport the incubation mixture comprised membrane suspension 0.2 ml, glucose (200 mM) 0.1 ml, $MgSO_4$ (40 mM) 0.1 ml, water or other component 0.1 ml, radiochemical substrate 0.5 ml. When phenazine methosulphate-ascorbate was used to support transport, the incubation mixture comprised membrane suspension 0.2 ml, phenazine methosulphate (1 mM) 0.1 ml, sodium ascorbate (200 mM, pH 7.1 containing 40 mM $MgSO_4$) 0.1 ml, water or other component 0.1 ml, radiochemical substrate 0.5 ml. Initial rates of uptake were measured in duplicate from four 0.2-ml samples taken during the first 2 min and are expressed as nmol/min per g protein. For assays of Cs^+ transport, valinomycin was added immediately after the membrane suspension, at the concentrations shown in the legends to figures. Initial rates were measured in duplicate from four 0.2-ml samples taken during the first minute.

Extraction of accumulated material: Intracellular material was isolated using the cold-shock technique described previously [16].

Measurement of intracellular cell water: Intracellular cell water was determined from the ratio of wet weight to dry weight of a packed cell pellet, using tritiated sucrose as a non-permeating species to measure the extracellular pellet water.

Measurement of intravesicular water: Intravesicular water was estimated from the difference between the total water in a vesicle pellet measured using the permeating species tritiated H_2O and the extracellular water measured using the non-permeating species [U-^{14}C]sucrose.

Measurement of respiration-driven proton translocation. The method used was similar to that described by Scholes and Mitchell [17]. Bacteria were harvested, washed in 10 mM glycylglycine, pH 7.1, and resuspended in this buffer containing 0.4 M sucrose, at a cell density of approx. 15 mg dry wt./ml. The reaction vessel consisted of a glass chamber into which the pH and reference electrodes were fitted. This chamber, volume 6 ml, was maintained

at a constant temperature of 25°C by means of a circulating-water jacket, and the contents were mixed by a magnetic stirrer. The chamber was filled with 300 mM KCl (6 ml) or varying proportions of KCl and KSCN and the cell suspension (2 ml) was introduced by syringe through a small inlet tube in the stopper. Carbonic anhydrase was added at a final concentration of 40 μg enzyme/ml to catalyse bicarbonate/CO_2 equilibration. Residual oxygen was rapidly utilized, and the pH was adjusted to the range 7.0—7.2 using standard solutions of HCl and KOH in 0.1 M KCl. The scale was calibrated by additions of 50 mM HCl in 0.1 M KCl made anaerobic by flushing with oxygen-free nitrogen. Oxygen pulses were given as CO_2-free, air-saturated 150 mM KCl, which contained 0.47 ng atom dissolved oxygen per μl at 760 mm Hg and 25°C [18].

Respiration studies. Rates of oxygen utilization by bacteria were measured at 25°C using a Beckman oxygen meter. Cells (2.5 mg) suspended in 50 mM HEPES/Tris, pH 7.1, were incubated with the inhibitors for 10 min before the addition of glucose at a final concentration of 3.3 mM.

Rates of oxygen utilization by vesicles prepared and suspended in HEPES/Tris, pH 7.1, were measured at 25°C using a Metabolic Oxygen Meter (Scientific and Educational Aids (Windsor) Ltd., England). Vesicles (1.2 mg protein) suspended in 21 mM HEPES/Tris containing 4 mM $MgSO_4$ were incubated with the inhibitors for 5 min before the addition of glucose at a final concentration of 20 mM.

Preparation of membrane fragments from P. fluorescens for ATPase assay. Cells were harvested, washed twice and resuspended in 20 ml of 30 mM Tris · HCl buffer, pH 8.0, at 4°C, at an approximate density of 20 mg dry wt./ml. Cell disruption was achieved by passage of this suspension through a pre-cooled French pressure cell operated at 34.4 MP. The extract was centrifuged for two 10-min periods at 7700 × g, the supernatant fluid was decanted and was ultracentrifuged at 100 000 × g for 1 h at 4°C to sediment the membranes. The pellet was resuspended in 10 mM Tris · HCl, pH 8.0, containing 50% (v/v) glycerol, and was stored on ice.

Assay of ATPase activity. ATPase activity was assayed as described previously [19] using assay system 1.

Analytical methods. (a) Chromatography: The following separation systems using Whatman No. 1 paper were used: (1) butan-1-ol/acetic acid/water (12 : 3 : 5, by vol.) ascending; (2) ethanol/ammonia/water (80 : 4 : 16, by vol.) ascending.

(b) Electrophoresis: This was performed at 3000 V for 45 min using Whatman No. 1 paper and a solvent system (3) comprising acetic acid/formic acid/water (15 : 5 : 180, by vol.), pH 1.9.

Unlabelled amino acids used as markers were made visible by spraying the paper with a solution of ninhydrin in acetone (0.5%, w/v), followed by heating in an oven.

(c) Membrane protein: Portions (1 ml) of membrane suspensions were mixed with 4 ml of 1 M NaOH was were boiled for 10 min in stoppered tubes. On cooling, 1-ml samples were removed for protein estimation using the Folin-Ciocalteau reagent [20].

(d) Inorganic phosphate: This was determined by the method of Allen [21].

Radiochemical techniques. Radioactivity on filters from transport assays, and on chromatograms, was counted using the system described previously [16]. For double-isotope counting, as in the measurement of the intravesicular volume, a fluid of the following composition was used: 60 g naphthalene, 10 g 2(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole, 100 ml methanol, 20 ml ethanediol, dioxan to 1 l. To minimize quenching, technical grade dioxan was refluxed for 40 min over sodium metal to reduce peroxides present. The dioxan was then collected by distillation and was stored in the dark, under nitrogen, at 4°C.

Chemicals. All amino acids were of the L-form except glycine. α-Aminoisobutyrate and 6-deoxyglucose were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Antimycin A, HOQNO, CCCP, *p*-chloromercuribenzoate, valinomycin, phenazine methosulphate, 2-deoxyglucose, pyruvate kinase (salt-free powder), carbonic anhydrase, lysozyme, DNAase, phospho(enol)pyruvate and ATP were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Dicyclohexylcarbodiimide and sodium succinate were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. TPMPBr and DDACl were obtained from K and K Laboratories Inc., Plainview, New York, U.S.A. 2,4-Dinitrophenol was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. TMPD was obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. and was recrystallized from hot water.

Radiochemicals. 2-Aminoiso[1-^{14}C]butyric acid, [U-^{14}C]sucrose, [6,6′(n)-$^{3}H_2$]sucrose, $^{3}H_2O$ and ^{137}Cs (as CsCl) were supplied by the Radiochemical Centre, Amersham, U.K.

Results

Experiments with cells

Washed cell suspensions of *P. fluorescens*, grown on a complex medium, accumulated ^{14}C-labelled material when exposed to α-aminoiso[1-^{14}C]butyrate (Fig. 1). Extraction of this material by the cold shock technique described in Materials and Methods, followed by chromatography in solvent systems 1 and 2 and electrophoresis in solvent system 3 indicated a single component, migrating with authentic α-aminoisobutyrate. Determination of intracellular water volume yielded a value of 1.7 ml/g dry wt. The plateau in uptake (Fig. 1a) thus represents an intracellular concentration of α-aminoisobutyrate 320-fold higher than the extracellular concentration. In experiments in which the rates of α-aminoisobutyrate transport into cells not containing α-aminoisobutyrate and cells pre-loaded with α-aminoisobutyrate were compared, this constant value for α-aminoisobutyrate accumulation was shown to be the result of a steady state.

Studies of the variation in the initial rate of α-aminoisobutyrate transport in response to α-aminoisobutyrate concentration indicated the operation of two systems that had apparent K_m values of 3.2 and 105 μM, respectively, the corresponding values for the V being 0.6 and 3.4 μmol/min per g dry wt. These data were obtained in the phosphate-buffered system. In the HEPES/Tris system the value for the low K_m component was 3.6 μM while the K_m value for the second component was measured as 60 μM.

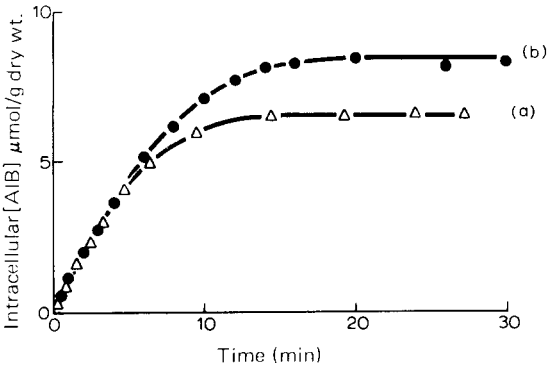

Fig. 1. Time course of α-aminoisobutyrate transport by *P. fluorescens*. Transport was assayed as described in Materials and Methods. α-Aminoiso[1-^{14}C]butyrate (AIB) was present at a final concentration of 11.7 μM. In a the organism was suspended in 0.067 M KH_2PO_4, pH 7.1, containing 0.1% (w/v) $MgSO_4$ and in b was suspended in 0.05 M HEPES/Tris, pH 7.1.

The specificity of the system operating at low α-aminoisobutyrate concentrations was investigated (Table I), the results indicating that α-aminoisobutyrate enters the organism on a system normally transporting the aliphatic neutral amino acids shown here. Further experiments revealed that alanine was a competitive inhibitor with a K_i of 3.5 μM.

Variation of the ionic composition of the medium in which transport was assayed revealed that α-aminoisobutyrate uptake could proceed when the organism was suspended in a HEPES/Tris-buffered system, with no apparent requirement for any inorganic ion (Fig. 1b); the amount accumulated at the plateau represents a 420-fold concentration of α-aminoisobutyrate. The time

TABLE I

SPECIFICITY OF α-AMINOISOBUTYRATE TRANSPORT BY CELLS AND MEMBRANE VESICLES OF *P. FLUORESCENS*

In experiments with cells, transport was measured in the HEPES/Tris buffer system. Amino acids, at a final concentration of 5 μM (except serine, 10 μM) were added simultaneously with α-aminoiso[1-^{14}C]-butyrate (final concentration 1 μM). The results were compiled from two experiments in which the rates of transport measured in the absence of any presumed competitor were 0.21 and 0.18 μmol/min per g dry wt. Membrane vesicles were prepared and assayed in phosphate-buffered medium. The phenazine methosulphate-ascorbate assay system was used and amino acids, at a final concentration of 8.3 μM were added 20 s prior to α-aminoiso[1-^{14}C]butyrate (final concentration 2 μM). The rate of transport measured in the absence of any presumed competitor was 19 nmol/min per g protein.

Addition	Relative initial rates of transport	
	Cells	Vesicles
None	100	100
Alanine	8	18
Glycine	14	60
Serine	8	91
Valine	12	96
Phenylalanine	95	93
Tryptophan	99	98

TABLE II

EFFECT OF VARIOUS INHIBITORS ON THE TRANSPORT OF α-AMINOISOBUTYRATE INTO *P. FLUORESCENS*

Transport was measured in the HEPES/Tris system and inhibitors were added at the beginning of the 10 min preincubation period prior to α-aminoiso[1-^{14}C]butyrate addition. The control values varied between 0.27 and 0.46 μmol/min per g dry wt. using 1 μM, and 2.55 and 3.56 μmol/min per g dry wt. using 150 μM α-aminoisobutyrate.

Inhibitor	Concentration (mM)	Relative initial rate of transport	
		1 μM α-aminoisobutyrate	150 μM α-aminoisobutyrate
NaN_3	5	26	29
	30	0	0
KCN	1	32	84
	10	37	80
DDAC1	1	60	50
	10	6	3
TPMBr	1	90	78
	2	47	43
CCCP	0.04	47	46
	0.10	5	7
Dinitrophenol	0.5	79	77
	2.0	12	16

courses illustrated were obtained with independent batches of the organism. In an experiment in which a single batch of organism was divided, one portion assayed in the phosphate-buffered system, the other in the HEPES/Tris system, identical initial rates of α-aminoisobutyrate transport were detected. As already noted the K_m values for the two components for α-aminoisobutyrate transport had similar values in the two systems.

Investigation of the energy-coupling mechanism for α-aminoisobutyrate transport employed α-aminoisobutyrate at 1 and 150 μM external concentrations, under conditions of partial and near-complete inhibition, so that any difference in energy coupling of the two systems might be detected. Using 1 μM α-aminoisobutyrate approx. 80% entered by the low K_m component, and using 150 μM α-aminoisobutyrate approx. 70% entered by the high K_m component. Transport was sensitive to electron transport inhibitors, such as azide, to uncouplers and to permeant cations (Table II). Control experiments indicated that the Cl^- and Br^- anions of dibenzyldimethylammonium and methyltriphenylphosphonium, respectively, were not responsible for the inhibition of α-aminoisobutyrate transport. Valinomycin, up to concentrations of 20 μg/ml also had no effect on α-aminoisobutyrate transport by *P. fluorescens*. Antimycin A and 2-heptylhydroxyquinoline-*N*-oxide, at final concentrations of 182 and 193 μM, respectively, caused no inhibition of α-aminoisobutyrate uptake or O_2 consumption by *P. fluorescens*. KCN (1 mM) and NaN_3 (30 mM) inhibited glucose-supported respiration by 44 and 100%, respectively. When cells were treated with the energy transfer inhibitor DCCD no inhibition of α-aminoisobutyrate transport was observed (Table III), and as shown, when

TABLE III

EFFECT OF DICYCLOHEXYLCARBODIIMIDE ON MEMBRANE-BOUND ATPase AND TRANSPORT OF α-AMINOISOBUTYRATE INTO *P. FLUORESCENS*

For (a) washed cell suspensions (≈20 mg dry wt./ml) in Tris · HCl buffer (30 mM, pH 8.0) were treated as indicated at 25°C on a shaking water bath and subsequently washed twice in the same buffer. Membrane fragments were prepared and assayed for ATPase as described in Materials and Methods. The results were compiled from duplicate experiments. For (b) washed cell suspensions (2—2.5 mg dry wt./ml) in HEPES/Tris (50 mM, pH 7.1) were incubated at 25°C with the indicated addition. Samples were removed and assayed for α-aminoisobutyrate transport in the HEPES/Tris system. The final concentration of α-aminoisobutyrate was 1 μM in Expt. 1 and 150 μM in Expt. 2.

(a) Effect on ATPase

Addition to cell suspension	Time of incubation (min)	Specific activity of ATPase in membrane fraction (μmol P_i released/min per mg protein)
Methanol, 1% (v/v)	0	0.23
	90	0.28
Dicyclohexylcarbodiimide, 816 μM	60	0.09
	90	0.02

(b) Effect on transport

Addition to cell suspension	Time of incubation (min)	Initial rate of α-aminoisobutyrate transport (μmol/min per g dry wt.)	
		Exp. 1	Expt. 2
Methanol, 1% (v/v)	0	0.28	2.76
	90	0.27	2.72
Dicyclohexylcarbodiimide, 816 μM	0	0.27	2.68
	90	0.29	2.65

membrane fragments were prepared from such cells, <90% inactivation of ATPase was observed.

As expected for a strictly aerobic organism, these results indicated that transport was probably supported by some uncoupler-sensitive intermediate or state prior to ATP itself. Evidence was thus sought for the existence of a respiration-driven, electrogenic proton extrusion mechanism. Experiments indicating proton extrusion by an anaerobic suspension of *P. fluorescens* in response to a pulse of air-saturated KCl, are illustrated in Fig. 2. As in other bacteria proton extrusion was stimulated in the presence of a permeant ion, in this case SCN^- was used (Fig. 2a). The maximum $\rightarrow H^+/O$ ratio detected, by extrapolation of semilog plots of ΔpH decay gave a value of 3.6 in 125 mM KSCN. This proton extrusion was sensitive to CCCP, enhancing the decay of the ΔpH (Fig. 2b). Experiments in which α-aminoisobutyrate (0.8 mM) was included in the incubation mixture for $\rightarrow H^+/O$ determination, demonstrated no appreciable change in decay of ΔpH, in contrast to the stimulation observed by West and Mitchell [22] when lactose was added to similar suspensions of *Escherichia coli*. The inability of α-aminoisobutyrate to elicit a more rapid decay of ΔpH, or induce the formation of ΔpH when added under anaerobic conditions, did not allow the possibility of a proton-symport mechanism

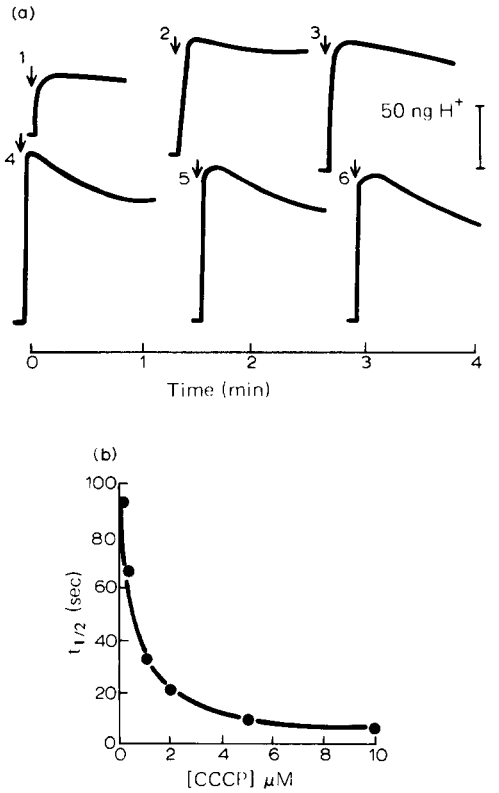

Fig. 2. Respiration-driven proton translocation by *P. fluorescens*. (a) The experiments were performed as described in Materials and Methods. Oxygen (47 ng atoms) was added as air-saturated 150 mM KCl at the time indicated by the arrows. The conditions were as follows: (1), 300 mM KCl; (2), 250 mM KCl and 50 mM KSCN; (3), 200 mM KCl and 100 mM KSCN; (4), 175 mM KCl and 125 mM KSCN; (5), 150 mM KCl and 150 mM KSCN; (6), 75 mM KCl and 225 mM KSCN. (b) The effect of CCCP was determined using the conditions described for experiment 4 above (a). CCCP was added prior to the oxygen pulse. Semi-logarithmic plots of the $\rightarrow H^+/O$ ratio versus time were constructed and the half-time of decay of the proton pulse ($T_{1/2}$) was calculated.

to be excluded since control experiments have indicated that, under these conditions, α-aminoisobutyrate required approx. 20 min to equilibrate across the membrane.

Experiments with membrane vesicles

When membrane vesicles were prepared in phosphate- or HEPES/Tris-buffered media as described in Materials and Methods, the uptake of α-amino-isobutyrate was observed (Fig. 3a). The two procedures yielded membrane vesicles which had essentially similar properties, suggesting that no major ionic requirement, such as for Na^+ or K^+, was necessary for α-aminoisobutyrate transport. Such transport was, however, clearly dependent upon a supply of reducing equivalents (Figs. 3a and 3b). The following relative initial rates of α-aminoisobutyrate transport were observed when membrane vesicles were prepared and assayed in phosphate-buffered medium with different electron

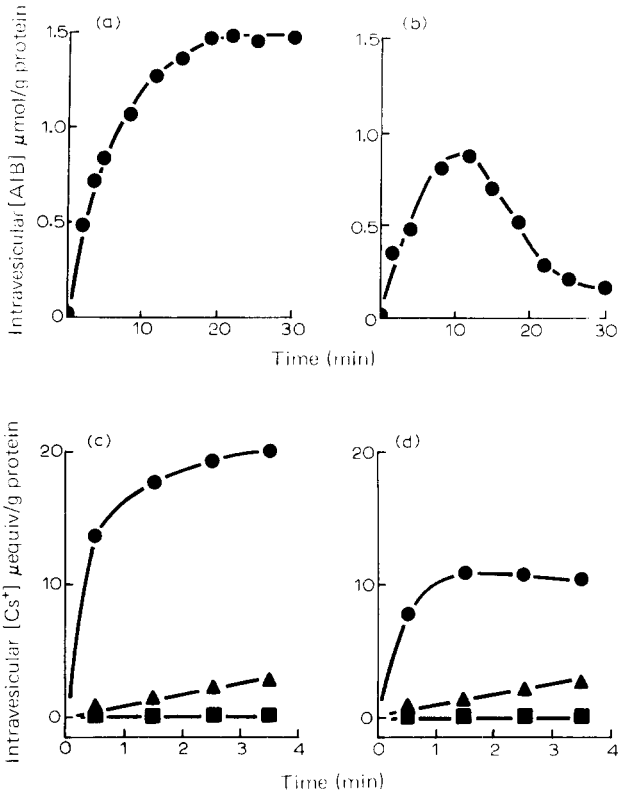

Fig. 3. Time courses of α-aminoisobutyrate transport and valinomycin-dependent caesium transport by membrane vesicles prepared from *P. fluorescens*. Membrane vesicles were prepared and assayed in phosphate-buffered medium in a and b, and HEPES/Tris in c and d. For α-aminoisobutyric acid (AIB) transport (final concentration 20 μM) the assay conditions were (a) 20 mM ascorbate, 0.1 mM phenazine methosulphate, no pre-incubation; (b) 10 mM ascorbate, 0.1 mM phenazine methosulphate, 5 min pre-incubation. For caesium transport (final concentration 2.5 mM), valinomycin was added at a final concentration of 5 μg/ml and transport was supported by phenazine methosulphate-ascorbate in c and glucose in d. ●———●, complete assay mixture; ▲———▲, valinomycin omitted; ■———■, electron donor omitted.

donors (all at 20 mM except phenazine methosulphate which was 0.1 mM): Sodium ascorbate plus phenazine methosulphate, 100; sodium ascorbate plus TMPD, 47; sodium succinate, 20; sodium malate, 44; glucose, 72; 6-deoxyglucose, 72; 2-deoxyglucose, 72; no addition, 5. Phenazine methosulphate alone caused no stimulation of α-aminoisobutyrate transport, while ascorbate alone caused a slight stimulation (19% of the value when added in the presence of phenazine methosulphate).

As shown (Fig. 3a), α-aminoisobutyrate accumulation reached a plateau which, as demonstrated by suitable preloading experiments, was the result of the operation of a steady-state system. Measurement of the intravesicular water, using tritiated water as the permeating species and [^{14}C]sucrose as the non-permeating species, yielded a value of 0.7 μl/mg vesicle dry wt. Using this value then, a typical concentration ratio achieved for α-aminoisobutyrate was 10- or 60-fold depending whether the HEPES/Tris- or phosphate-buffered system was employed.

Studies on the kinetics of α-aminoisobutyrate transport into membrane vesicles produced by both procedures demonstrated, using a conventional double-reciprocal plot, that only a single component, with a K_m of 46 μM was involved. The V for α-aminoisobutyrate transport was somewhat higher (210 versus 100 nmol/min per g protein) for membrane vesicles prepared and assayed in the phosphate-buffered system compared with those prepared and assayed in the HEPES/Tris-buffered system. The value of the K_m suggested that the low K_m component detected in the intact organism was not present in membrane vesicles prepared from such cells. This was confirmed with the demonstration (Table I) of a marked difference in specificity between the system detected in vesicles and that of the low K_m component in cells, notably the diminished efficiency of valine and serine as competitors.

The assay medium for both types of membrane vesicle contained Mg^{2+} (4 μequiv./ml). α-Aminoisobutyrate transport could be detected in the absence of Mg^{2+}, but 4 mM $MgSO_4$ was required to maximally stimulate transport (1.6-fold and 2.4-fold when energized by glucose or phenazine methosulphate-ascorbate, respectively). Glucose-supported respiration by membrane vesicles prepared and assayed in HEPES/Tris was also stimulated maximally by 4 mM $MgSO_4$, approx. 1.6-fold.

The sensitivity of α-aminoisobutyrate to a range of inhibitors was studied with both types of membrane vesicle preparation. The results for membrane vesicles prepared and assayed in HEPES/Tris are presented (Table VI) as part of a comparative study with valinomycin-dependent Cs^+ transport (see below). Transport of α-aminoisobutyrate was also sensitive to *p*-chloromercuribenzoate (<0.5 μM) and iodoacetamide (10 mM) which caused 40 and 20% inhibition, respectively. In addition, transport of α-aminoisobutyrate was sensitive to permeant cations; dibenzyldimethylammonium (10 mM) and methyltriphenylphosphonium (3 mM) caused greater than 90% inhibition of transport. K^+ (10 μequiv./ml), in the presence of valinomycin, also inhibited α-aminoisobutyrate transport; the concentration of valinomycin required to produce ≈80% inhibition was 5 μg/ml.

Glucose-supported respiration by membrane vesicles prepared and assayed in HEPES/Tris was sensitive to Antimycin A (182 μM), 2-heptyl-4-hydroxyquinoline-*N*-oxide (193 μM), KCN (10 mM) and NaN_3 (30 mM) which caused 80, 38, 85 and 37% inhibition, respectively. The sensitivity of transport and respiration in membrane vesicles to 2-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A presumably reflects the change is accessibility of the membrane to these compounds. Similar considerations apply with respect to valinomycin.

Role of a membrane potential in α-aminoisobutyrate transport

Inhibition of α-aminoisobutyrate transport into cells and membrane vesicles by dibenzyldimethylammonium and methyltriphenylphosphonium ions, together with the inhibition by valinomycin/potassium observed with membrane vesicles suggested a role for a membrane potential in both systems. To assess the magnitude of the membrane potential, and compare the sensitivity towards uncouplers and electron transport inhibitors of its generation with the sensitivity of α-aminoisobutyrate transport to the same agents, we

have characterized valinomycin-induced Cs^+ transport into membrane vesicles. Such transport was readily observed with vesicles prepared and subsequently assayed in the HEPES/Tris system (Fig. 3), chosen to avoid the effects of K^+ present in the phosphate-buffered system. Uptake of Cs^+ was dependent upon valinomycin and the presence of a suitable electron donor (Figs. 3c and 3d), reaching a plateau value that, when transport was supported by phenazine methosulphate-ascorbate, decayed after approx. 5 min, whereas, when supported by glucose, was maintained for longer periods. This was probably related to the diminished supply of reducing equivalents from this auto-oxidizable couple as suggested by previous data for α-aminoisobutyrate transport (Figs. 3a and 3b). Kinetic studies revealed that Cs^+ transport was a saturable process, showing an apparent K_m for CsCl of 0.35 or 0.42 mM with a V of 14.5 or 12.8 μequiv. Cs^+/min per g protein when the valinomycin concentration was 5 or 0.5 μg/ml, respectively, and the vesicle density was 1.84 and 1.92 mg protein/ml, respectively. These results were obtained with two independent preparations in which transport was supported by phenazine methosulphate-ascorbate. Such transport was also saturable with respect to valinomycin, showing an apparent K_m of 0.2 μg/ml when the CsCl concentration was 1 mM, the concentration of membrane vesicles was 1.61 mg protein/ml, and transport was supported by phenazine methosulphate-ascorbate. The addition of increasing amounts of valinomycin above 0.5 μg/ml caused a

TABLE IV

EFFECT OF THE CONCENTRATION OF VALINOMYCIN AND CAESIUM ON THE MAGNITUDE OF THE MEMBRANE POTENTIAL GENERATED BY MEMBRANE VESICLES PREPARED FROM *P. FLUORESCENS*

Membrane vesicles were prepared and assayed in HEPES/Tris. Phenazine methosulphate-ascorbate was used to support transport in a and glucose in b. $^{137}CsCl$ was added at a final concentration of 1 mM in a and valinomycin was present at a concentration of 0.5 μg/ml in b.

(a) Effect of valinomycin

Concentration (μg/ml)	Membrane potential (mV)	
	Expt. 1	Expt. 2
0.125	—	57
0.250	58	58
0.500	57	55
0.750	48	—

(b) Effect of caesium

Concentration (mM)	Membrane potential (mV)	
	Expt. 1	Expt. 2
1.0	45	51
0.5	81	85
0.1	92	101
0.05	95	110
0.02	—	103
0.01	—	107

TABLE V

A COMPARISON OF α-AMINOISOBUTYRATE AND CAESIUM TRANSPORT BY MEMBRANE VESICLES PREPARED FROM *P. FLUORESCENS*

Membrane vesicles were prepared and assayed in HEPES/Tris using α-aminoisobutyrate or Cs^+ at final concentrations of 30 μM and 1 mM, respectively. For assays of Cs^+ transport valinomycin was present at a concentration of 0.5 μg/ml. Initial rates of uptake are expressed as μequiv. or μmol/min per g protein for Cs^+ and α-aminoisobutyrate respectively. Similarly, the amounts of substrate accumulated are expressed as μequiv. Cs^+/g protein and μmol α-aminoisobutyrate/g protein.

Substrate	Assay system	Initial rate of transport	Amount of substrate accumulated	Intravesicular substrate concentration (mM)	Concentration ratio
$^{137}CsCl$	Phenazine methosulphate-ascorbate	9.00	13.6	11.3	11.6
α-Aminoiso[1-^{14}C]butyrate	Phenazine methosulphate-ascorbate	0.040	0.35	0.29	9.9
$^{137}CsCl$	Glucose	5.50	10.9	9.1	9.3
α-Aminoiso[1-^{14}C]butyrate	Glucose	0.022	0.28	0.23	7.8

progressive decrease in the initial rate of Cs^+ transport (Cs^+ concentration 1 mM) and in the steady-state level accumulated. However, although the initial rate of Cs^+ uptake at concentrations of valinomycin lower than 0.5 μg/ml was proportional to the valinomycin concentration, uptake reached the same steady-state level and hence the magnitude of the potential generated was constant (Table IV (a)). This value did however vary with the external Cs^+ concentration. At a valinomycin concentration of 0.5 μg/ml, the maximum value of 110 mV was obtained for the membrane potential when the Cs^+ concentration was 0.05 mM or lower and glucose was used to support transport (Table IV (b)). Attempts to measure the maximum membrane potential using the phenazine methosulphate-ascorbate system were unsuccessful as the value obtained was also governed by the time at which the supply of reducing equivalents was exhausted. This factor was significant in these experiments since at low Cs^+ concentrations longer time periods (approx. 20 min) were required before the Cs^+ distribution reached equilibrium with the membrane potential. These problems were circumvented by using glucose as the electron donor.

Valinomycin-dependent Cs^+ transport was inhibited by K^+; 1 mM KCl caused 40% inhibition of Cs^+ transport (2.5 mM CsCl). A comparison of phenazine methosulphate-ascorbate and glucose-supported α-aminoisobutyrate and Cs^+ transport (Table V) showed that the amount of both substrates accumulated in the presence of phenazine methosulphate-ascorbate was greater than in the presence of glucose, and that the concentration ratios achieved in the steady state were similar for each substrate. A comparison of the effect of uncouplers and electron transport inhibitors on the initial rates of Cs^+ and α-aminoisobutyrate transport (Table VI) also revealed very similar sensitivities of both systems to the inhibitors tested. In addition, both systems were inhibited by

TABLE VI

EFFECT OF UNCOUPLERS AND ELECTRON TRANSPORT INHIBITORS ON THE TRANSPORT OF CAESIUM AND α-AMINOISOBUTYRATE INTO MEMBRANE VESICLES PREPARED FROM *P. FLUORESCENS*

Membrane vesicles were prepared and assayed in HEPES/Tris, using the phenazine methosulphate-ascorbate assay system. For assays of caesium transport, valinomycin was added at a final concentration of 0.5 μg/ml. The vesicles were incubated with the inhibitors for 5 min prior to the addition of radiochemical substrate, $^{137}CsCl$ (1 mM) or α-aminoiso[1-^{14}C]butyrate (30 μM). The results are compiled from four experiments in which the control values were between 2.9 and 10.2 μequiv./min per g protein for caesium transport and between 51 and 70 nmol/min per g protein for α-aminoisobutyrate transport.

Addition	Concentration (mM)	Relative initial rate of transport	
		Caesium	α-Aminoisobutyrate
None	—	100	100
Methanol	1% (v/v)	95	94
CCCP	0.01	10	20
Dinitrophenol	1.0	59	49
NaN_3	30	43	20
HOQNO	0.019	90	88
Antimycin A	0.182	10	8
KCN	10	19	12

greater than 90% by the permeant cations dibenzyldimethylammonium (10 mM) and methyltriphenylphosphonium (3 mM).

Discussion

α-Aminoisobutyrate transport by *P. fluorescens* has been shown to be mediated by two components, each capable of saturation with, and possessing different apparent K_m values for α-aminoisobutyrate. A non-linear, double-reciprocal plot of the initial rate of uptake versus substrate concentration is characteristic of amino acid transport by many microorganisms, e.g. proline and other amino acids in *Pseudomonas aeruginosa* [23], alanine and glycine in *E. coli* [24] and serine in *E. coli* [25]. α-Aminoisobutyrate was accumulated without chemical modification, thus eliminating a group translocation mechanism for α-aminoisobutyrate transport and complicating effects due to metabolism. Studies on the specificity of α-aminoisobutyrate transport via the low K_m transport system (Table I) suggest that α-aminoisobutyrate was transported by a component serving a range of structurally related amino acids comprising alanine, glycine, serine and valine. Similar systems have been reported previously, e.g. separate transport systems exist for aliphatic, basic and aromatic amino acids in *P. aeruginosa* [26], and at least nine different systems exist in *E. coli* including one for alanine, glycine, serine and threonine [27]. The overlapping specificity of such multiple amino acid transport systems accounts for the existence of more than a single component mediating the uptake of certain amino acids.

α-Aminoisobutyrate was accumulated by membrane vesicles prepared from *P. fluorescens* when either phenazine methosulphate or glucose was added to stimulate transport. The ability of glucose to support transport reflects the presence of an extracellular, membrane-bound glucose oxidase in this organism [28]; 2-deoxyglucose and 6-deoxyglucose are probably substrates for this enzyme as has been demonstrated in *P. aeruginosa* [16]. α-Aminoisobutyrate uptake by membrane vesicles was mediated by only a single component with a K_m value similar to that obtained for the second component of α-aminoisobutyrate transport by cells of *P. fluorescens*. The specificity of α-aminoisobutyrate transport by membrane vesicles also differed from that of the parent cells being severely inhibited by alanine, but showing decreased sensitivity to glycine and a marked insensitivity to serine and valine (Table I). This altered specificity supports the kinetic evidence that α-aminosiobutyrate transport by membrane vesicles was via the high K_m component, the low K_m transport system being lost or inactivated during vesicle preparation. Similarly, despite the fact that *P. fluorescens* was able to transport Cs^+ (data not shown), presumably via the K^+ transport system, vesicles were unable to accumulate Cs^+ until valinomycin was added, demonstrating a similar loss of the Cs^+ transport system. It has previously been shown that transport of certain amino acids and sugars by a number of Gram-negative organisms is also sensitive to an osmotic shock treatment, such as that employed in vesicle preparation. Such treatment causes the release from the periplasmic space of proteins which can specifically bind the solute whose transport has been impaired by the shock treatment [29—31]. Comparative studies of the energy-coupling mechanism for the

transport of solutes via 'shockable' and 'non-shockable' transport systems [32,33] have suggested that different mechanisms exist for each type of system; transport by 'non-shockable' systems being driven by an energized membrane state, whereas transport by 'shockable' systems showed an obligatory requirement for 'phosphate bond energy'. The results presented here, however, suggest that although the low K_m component for α-aminoisobutyrate transport was lost during vesicle preparation, a similar mechanism of energy coupling operated for α-aminoisobutyrate transport via both components. Although KCN may have a differential effect on the two transport systems, transport at 1 and 150 μM α-aminoisobutyrate shows similar sensitivity to a range of inhibitors (Table II) and was also not impaired, at either concentration, in the presence of dicyclohexylcarbodiimide which inactivated the membrane-bound ATPase, and hence prevented ATP synthesis in this strictly aerobic organism (Table III). A direct role for the involvement of ATP in the energy-coupling mechanism for the high K_m system was also eliminated by the studies of the energy-coupling mechanism in membrane vesicles, since transport occurred without addition of ADP and P_i.

The observation that vesicles retained the ability to accumulate α-aminoisobutyrate, albeit with a diminished maximum velocity, when phosphate-buffered medium was replaced by HEPES/Tris suggested that gradients of inorganic ions were not involved in the energy-coupling mechanism. This is supported by the experiments with cells in which identical initial rates of α-aminoisobutyrate transport and similar K_m values were obtained when cells were suspended in sodium, potassium phosphate or HEPES/Tris. However, respiring *P. fluorescens* have been shown to extrude protons (Fig. 2), the maximum detectable $\rightarrow H^+/O$ ratio of 3.6 being measured in the presence of 125 mM KSCN. Rather higher values of about 6.5 have previously been measured for the $\rightarrow H^+/O$ ratio of *Pseudomonas ovalis* Chester also oxidizing endogenous substrates [34]. Whether the diminished magnitude of the $\rightarrow H^+/O$ ratio is significant has not yet been established, but these observations show that an uncoupler-sensitive proton-motive force can be generated by *P. fluorescens* under these conditions. We have not yet measured the size of this force.

The studies of the inhibition of α-aminoisobutyrate transport in cells and vesicles, in particular the sensitivity of transport to permeant cations in both systems, suggests the involvement of at least the membrane potential component of this proton-motive force in α-aminoisobutyrate uptake. Comparative studies of α-aminoisobutyrate and Cs^+ transport in membrane vesicles confirm this and show that the membrane potential generated by vesicles prepared and assayed in HEPES/Tris (approx. 100 mV, Table IV) was of sufficient magnitude to support the α-aminoisobutyrate concentration ratios measured in such vesicles (approx. 10-fold, Table V). The value for the Cs^+ concentration ratio presented in this table was not the maximum ratio of which this preparation was capable since the Cs^+ concentration employed in these experiments was 1 mM and, as shown in Table IV, the membrane potential will be approximately twice this. An estimation of the homogeneity of vesicle preparations was made by measuring the latency of the ATPase, and such measurements suggest that about two-thirds of the vesicles prepared and assayed in

HEPES/Tris were correctly orientated (Stephenson, M.C., Midgley, M. and Dawes, E.A., unpublished). Since inverted or ruptured vesicles do not contribute to the transport activity but do contribute to the total vesicle protein, the values estimated for the initial rates of transport and amounts of substrate accumulated by such vesicles will be underestimates by a factor of about one-third as the values presented in Results have not been corrected. Similar errors in the measurement of the Cs^+ concentration will also cause underestimates of the magnitude of the membrane potential. However, since the number of inverted or ruptured vesicles was a constant factor in any one preparation, the comparative data for α-aminoisobutyrate and Cs^+ transport (Table V) remains valid.

Although we have not estimated the ΔpH component in membrane vesicles of *P. fluorescens* it has been shown with vesicles derived from *E. coli* that at external pH values of 7 and above, this component probably makes only a small contribution to the proton-motive force, its contribution increasing as the external pH was lowered [8]. Whether this is true for the *P. fluorescens* system remains to be established. Our results therefore suggest that α-aminoisobutyrate transport in *P. fluorescens* could occur via a proton-symport mechanism that responds to the membrane potential. Attempts to obtain direct evidence for such a mechanism were hampered by the very slow equilibration time for α-aminoisobutyrate under anaerobic conditions. Thus any resulting proton movements may have been indistinguishable from the experimentally observed slow baseline drift of external pH experienced in such experiments. Similar difficulties have been encountered measuring proton movements coupled to alanine uptake by *E. coli* [35]. In these experiments the rate and extent of alanine transport were increased by growing *E. coli* in a chemostat with alanine as both the sole carbon source and limiting substrate, which resulted in the selection of mutants with increased stoichiometries of proton : alanine uptake. Thus, in view of such difficulties and the characteristics we have described, we do not consider that the inability to demonstrate α-aminoisobutyrate-induced proton movements is sufficient to eliminate a proton-symport mechanism for α-aminoisobutyrate transport in this organism.

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