The Transport of α -Aminoisobutyrate into *Crithidia fasciculata*

By M. MIDGLEY

Department of Biochemistry, University of Hull, Kingston-upon-Hull, HU6 7RX, U.K.

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The transport of α -aminoisobutyrate into *Crithidia fasciculata* was characterized under aerobic and anaerobic conditions. Kinetic data for α -aminoisobutyrate transport were consistent with the operation of a single system of broad specificity that showed no marked dependence on Na^+ . Under anaerobic conditions α -aminoisobutyrate transport was inhibited by uncouplers such as 2,4-dinitrophenol, lipophilic cations such as methyltriphenylphosphonium ion and adenosine triphosphatase inhibitors such as dicyclohexylcarbodi-imide and NaN_3 . A working model in which α -aminoisobutyrate enters this organism by an H^+ -symport mechanism, the electrochemical gradient of protons being maintained by an H^+ -translocating adenosine triphosphatase on the cytoplasmic membrane, is proposed.

Transport of nutrients into Trypanosomatids has been characterized mainly from the point of view of the number and specificity of the components involved (e.g. Hampton, 1970; Southworth & Read, 1972; Manjra & Dusanic, 1972; Ruff & Read, 1974*a,b*). The major problem investigated in the present paper concerns the process by which metabolic energy is used to support active transport in the organism *Crithidia fasciculata*. To this end the transport of α -aminoisobutyrate has been characterized; in particular the sensitivity of this transport process to a range of inhibitors has been studied under anaerobic conditions. As pointed out by Harold (Pavlasova & Harold, 1969), the effect of inhibitors under these conditions cannot be attributed to interference with oxidative phosphorylation; thus the target of ATPase inhibitors or uncouplers will not be the mitochondrial system, but some component involved in the overall transport process. Current ideas on the mechanism of action of the inhibitors employed in this study have been discussed by Hamilton (1975), and also by Harold (1970, 1972).

Methods

Organism

The organism used in this study was *Crithidia fasciculata* obtained from Dr. B. A. Newton, The Molteno Institute, Cambridge, U.K.

Growth and maintenance of cultures

The organism was grown on the complex medium described by Cosgrove (1963), solidified for slopes, on which the organism was maintained by monthly

subculture, by the addition of 0.6% (w/v) Agar no. 3 (Oxoid). Running cultures (10ml in 50ml Erlenmeyer flasks) were grown aerobically at 25°C on a Gallenkamp shaking water bath. Subcultures were made by loop every 3 days, and such cultures were used as inocula (5%, v/v) for larger volumes of medium (300ml in 1-litre Erlenmeyer flasks) which were harvested after 17h incubation.

Preparation of washed-cell suspensions

To minimize contamination with haemin, cultures were cooled on ice immediately before harvesting and filtered under suction through Whatman no. 1 filter paper. The organism was subsequently harvested by centrifugation at 3500*g* for 10min at 4°C. The pellet was resuspended to a smooth suspension (200ml) in a washing fluid appropriate to the experiment performed, either in buffer 1 (49mm-NaCl/167mm- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4/3.3\text{mm-MgSO}_4$, pH7.4), buffer 2 (100mm-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid adjusted to pH7.4 with Tris base) or 327mm-pentaerythritol. This latter wash was used for the majority of the experiments described. The organism was washed twice, resuspended to the required cell density and stored on ice.

Measurements of cell mass

The quantity of organism used in an experiment was assessed by relating the A_{540} of the suspension at a suitable dilution in buffer 1 to the dry weight of the organism. A calibration curve relating these two parameters was obtained by sedimenting a known volume of a washed-cell suspension, of known A_{540} , for 10min at 3500*g*. The supernatant fraction was discarded and the centrifuge tube allowed to drain

Abbreviation used: ATPase, adenosine triphosphatase.

without disturbing the pellet. The pellet was then resuspended in water and quantitatively transferred to a tared glass bottle and then heated at 108°C until constant weight was achieved.

Measurement of cell water

The volume of water associated with a given weight of the organism was measured by comparing the wet to the dry weight of a pellet of the organism, with suitable correction for the extracellular water in the pellet, with haemoglobin as a non-penetrating marker (Min, 1965).

Transport studies

The general techniques and apparatus were as described previously (Midgley & Dawes, 1973) except that glass-fibre filters (Whatman GF/A) and ice-cold washes were employed throughout. The major system used in these studies was as follows: cell suspension (6–12 mg dry wt./ml) in 327 mM-pentaerythritol, 0.5 ml; 491 mM-pentaerythritol, 1.5 ml; buffer 2, 0.25 ml; 110 mM-glucose, 0.25 ml; transport substrate, 0.25 ml. The complete system minus transport substrate was preincubated for 10 min at 25°C before initiation of the assay. Aerobic assay conditions were obtained by incubation in a 25 ml Erlenmeyer flask in a Gallenkamp shaking water bath. In experiments concerning the energy-coupling mechanism initial rates of transport were measured in duplicate from four samples (0.5 ml) removed during the 10 min after initiation of the assay. Assays under anaerobic conditions were performed in a water-jacketed glass vessel fitted with a rubber sealing cap through which was passed N₂ made O₂-free by passage through Fieser's reagent (20 g of KOH, 2 g of sodium anthraquinone-2-sulphonic acid and 15 g of sodium dithionite dissolved in 100 ml of water). The temperature was maintained at 25°C and the contents were magnetically stirred. For studies of the energy-coupling mechanism, initial rates were measured in duplicate from four samples (0.5 ml removed), with a suitable syringe, during the 20 min after initiation of the assay. Solutions of transport substrates were freed from O₂ by gassing with N₂. For both the aerobic and anaerobic system, inhibitors were added as either aqueous or methanolic solutions and were present throughout the assay, including the 10 min preincubation period. Suitable controls were performed to assess any solvent effects. The α -aminoisobutyrate concentration in the assays concerning energy-coupling was 91 μ M (specific radioactivity 1 or 2 μ Ci/ μ mol). For studies of the kinetics of α -aminoisobutyrate transport assay periods were adjusted so that the rates of uptake were linear over the time of measurement. Best straight lines for double-reciprocal plots were drawn by using an un-

weighted linear-regression program on a Hewlett-Packard model 9810A calculator.

Extraction and identification of the intracellular product of α -aminoisobutyrate accumulation

Washed-cell suspensions in buffer system 1 were incubated aerobically in a 50 ml Erlenmeyer flask with α -aminoisobutyrate in the following system: cell suspension (6 mg dry wt./ml), 1 ml; water, 0.5 ml; 1 mM- α -aminoisobutyrate (2 μ Ci/ μ mol), 0.5 ml; 110 mM-fructose, 0.5 ml; buffer 1, 3.0 ml. In the first type of extraction procedure samples were taken as for transport assays and, after a washing on a filter, were treated with ice-cold 60% (w/v) ethanol (4 ml/filter). The filtrate was collected and concentrated by rotary evaporation. This procedure effectively removed all the radioactive material originally present on the filter. In the second type of extraction procedure organism was loaded as described and then cooled on ice and centrifuged at 6000g for 5 min at 4°C. The organism was washed once, by resuspension in ice-cold buffer 1, before being finally resuspended in water plus 2% (v/v) toluene at 25°C for 10 min. This toluene treatment released effectively all the radioactive material associated with the organism, which was then sedimented at 6000g for 5 min and the supernatant fraction concentrated by rotary evaporation. The identity of the accumulated compound was established by ascending chromatography with butan-1-ol/acetic acid/water (16:3:5, by vol.) and electrophoresis in acetic acid/formic acid (100%)/water (3:1:36, by vol.), pH 1.9. Whatman no. 1 paper was used for both separations. Electrophoresis was carried out at 3 kV for 45 min on a Shandon high-voltage electrophoresis apparatus. Standards were detected by spraying with a solution of ninhydrin in acetone (0.5%, w/v), followed by heating briefly in an oven at 108°C. Radioactivity was detected by cutting the sample lane into sections (1 cm $\times$ 4 cm) and measuring the radioactivity by scintillation counting.

Respiration studies

These were performed with a Beckman Oxygen Analyser model 777 in a water-jacketed chamber (3.2 ml) maintained at 25°C and the contents of which were magnetically stirred. The chamber was completely filled with air-saturated pentaerythritol (327 mM), and then buffer 2 (0.3 ml) was introduced with a syringe. Organism, between 1 and 2 mg dry wt. suspended in 327 mM-pentaerythritol, was introduced and respiration followed over 10–20 min. When inhibitors were added as methanolic solutions, e.g. uncouplers and carbodi-imides, suitable controls were performed to assess the effect of the solvent. The O₂ content of the incubation medium was measured by using the NADH/phenazine methosulphate method (Robinson & Cooper, 1970).

Measurement of glycolysis

This was assayed as acid production from glucose by using a Radiometer pH-stat assembly (titrator type TTT1; chart recorder type SBR2c; syringe burette type SBU1a) at 25°C. The assay mixture had the following composition: 405 mM-pentaerythritol, 5 ml; water, 0.8 ml; 1:10 dilution of buffer system 2, 0.5 ml; 15–25 mg dry wt. of the organism in 327 mM-pentaerythritol, 1 ml; 6.5 mM-antimycin A, in methanol, 7.5 μ l. This system was incubated for 10 min before the addition of 375 mM-glucose (0.2 ml). After a 1 min lag, acid production commenced and was linear for at least 12 min. The assay was conducted under a stream of N₂ and the titrant was 20 mM-NaOH in 327 mM-pentaerythritol. At the end of each assay the scale was calibrated by addition of 4 μ mol of HCl, equivalent to approximately 20% the full-scale deflection. When inhibitors were added these were present throughout the assay. Suitable controls were performed to allow for the effect of methanol added with some inhibitors.

Radiochemical techniques

The counting system described previously (Midgley & Dawes, 1973) was used throughout.

Materials

The following chemicals were from the supplier indicated. α -Aminoisobutyrate was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Cycloleucine, carbonyl cyanide *m*-chlorophenylhydrazone and *NN'*-dicyclohexylcarbodi-imide were from Sigma Chemical Co., London, U.K. Methyltriphenyl phosphonium bromide and dibenzyltrimethylammonium chloride were from K and K Laboratories, Plainview, NY, U.S.A. *NN'*-Di-isopropylcarbodi-imide was from Ralph Emmanuel, Wembley, Middx., U.K. 4-Chloro-7-nitrobenzofuran was from Serva, Feinbiochemica, Heidelberg, West Germany, Antimycin A was from Boehringer Corp., London, U.K., and α -amino[1-¹⁴C]isobutyrate was supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Preliminary experiments

When *C. fasciculata* growing exponentially in a complex medium was harvested, washed and resuspended in buffer 1 the uptake of α -aminoisobutyrate and 1-aminocyclopentane-1-carboxylic acid (cycloleucine) (both used at a concentration of 1 mM) was demonstrated. Initially uptake studies were performed aerobically and under such conditions the addition of glucose or fructose (both 10 mM) caused an approximate 3-fold stimulation of the initial rate of trans-

port. α -Aminoisobutyrate transport was selected for further study and subsequent experiments confirmed stimulation although this varied 2–5-fold with different batches of the organism and α -aminoisobutyrate accumulation often responded to a greater extent (e.g. Fig. 2).

In most biological systems α -aminoisobutyrate is considered to be non-metabolizable and, since knowledge of this aids the interpretation of the transport data presented here, the intracellular material accumulated by *C. fasciculata* on exposure to α -amino[¹⁴C]isobutyrate, for 3–4 h aerobically in the presence of fructose, was isolated and analysed by electrophoresis and chromatography as described in the Methods section. The isolation techniques employed yielded greater than 98% recovery of the accumulated material, which moved as a single component with a mobility, in the systems described in the Methods section, close to that of authentic α -aminoisobutyrate. Thus α -aminoisobutyrate was not metabolized by *C. fasciculata*. Measurement of the amount of water associated with a known weight of the organism, with suitable correction for the haemoglobin-permeable water space, yielded a value of 1.75 ml/dry wt. By using this value, and assuming the whole of the intracellular water was permeated by α -aminoisobutyrate, it was shown that large concentration gradients of α -aminoisobutyrate were established; e.g. when the organism was incubated with α -aminoisobutyrate (0.1 mM) an internal concentration of 12 mM was achieved under aerobic conditions in the presence of 10 mM-fructose. Owing to the decrease in external substrate concentration to 0.07 mM this corresponded to a concentration ratio of 170-fold. Thus an active-transport system operates for α -aminoisobutyrate uptake by this organism.

Effect of incubation medium composition on α -aminoisobutyrate transport

In order to investigate the mechanism of energy-coupling to α -aminoisobutyrate transport, especially the role of ion gradients and co-transport, a comparison was made of the ability of the organism to transport α -aminoisobutyrate when harvested and assayed in buffer 1 or 2 (composition as described in the Methods section). The assays were performed aerobically in the presence of fructose. When a single batch of organism was divided and the two halves subsequently harvested independently with these two buffers a marked inhibition was noted with organism suspended and assayed in buffer 2. Suitable controls established that the components of buffer 2 were not inhibitory *per se* and that organism harvested in this buffer and subsequently assayed in buffer 1 transported α -aminoisobutyrate at the same rate as organism handled in buffer 1 throughout. Systematic addition of combinations of the major components

Table 1. *Effect of incubation medium composition on α -aminoisobutyrate transport into Crithidia fasciculata*
Initial rates of transport were measured in duplicate over the 10 min period after addition of α -aminoisobutyrate (91 μ M) to organism harvested and suspended in buffer 2. The assays were performed under aerobic conditions in the presence of fructose (10 mM).

Addition	Concentration (mM)	α -Aminoisobutyrate transport (μ mol/per g dry wt. min)
None	—	0.02
Sorbitol	218	0.23
Mannitol	218	0.22
KCl	109	0.13
NaCl	109	0.14
Pentaerythritol	327	0.18
Lactose	218	0.21

of buffer 1 (Na^+ , K^+ , HPO_4^{2-} and Cl^-) to organism harvested in buffer 2 demonstrated no specific requirement for this stimulation of transport. In addition it was established that transport was stimulated by a range of organic compounds, namely lactose, mannitol, pentaerythritol and sorbitol. A series of experiments was conducted with these compounds, and also NaCl and KCl, in order to optimize such stimulation. For the organic compounds the range employed was 150–327 mM, and for the inorganic 27.5–275 mM. The optimum concentration of each compound was then used in a comparative study on a single batch of organism (Table 1). Such a non-specific effect probably reflects the requirement for an adequate osmolarity of the suspending medium, since this organism has been shown to possess a contractile vacuole, the activity of which has been related to the osmotic pressure of the suspending medium (Cosgrove & Kessel, 1958). In further experiments no additional stimulation of α -aminoisobutyrate transport was observed when KCl and/or NaCl, both at concentrations of 10 mM, were added to organism suspended in buffer 2, with mannitol (218 mM) or pentaerythritol (327 mM) as the major organic constituent. In both these systems (mannitol or pentaerythritol) the addition of 5 mM- CaCl_2 or 5 mM- MgCl_2 caused a partial inhibition (approx. 40%) of α -aminoisobutyrate transport. In the experiments so far quoted the effects described were noted on initial rates of α -aminoisobutyrate transport. When uptake was followed for 3 h the addition of 10 mM-NaCl or 0.5 mM-ouabain had no effect on the amount of α -aminoisobutyrate accumulated (65 and 41 μ mol/g dry wt. for the mannitol and pentaerythritol systems respectively, equivalent to at least 800-fold concentration gradients). Analysis by flame photometry of the reagents used in these assays indicated that in the absence of added Na^+ or K^+ their concentrations did not exceed

Fig. 1. *Double-reciprocal plot for the inhibition of α -aminoisobutyrate transport by phenylalanine*

Initial rates of α -aminoisobutyrate transport were measured in the presence (Δ) and absence ($\circ$) of 20 mM-phenylalanine. Transport was followed under aerobic conditions in the presence of 10 mM-glucose in the pentaerythritol/buffer 2 system. The competitor was added 10 s before α -aminoisobutyrate.

120 nequiv./ml. As noted previously, when assayed in buffer 1 either 10 mM-glucose or -fructose caused a marked stimulation of α -aminoisobutyrate transport. Similar findings were made with organisms assayed in buffer 2 plus 327 mM-pentaerythritol. However, when mannitol was employed then α -aminoisobutyrate transport, in the absence of added glucose or fructose, was stimulated to values similar to those found in the fructose/glucose/pentaerythritol system, suggesting metabolism of mannitol was occurring. Thus the majority of the subsequent experiments were conducted with the pentaerythritol/buffer 2 system. Further experiments established that similar transport rates were measured if the organism was harvested and stored in 327 mM-pentaerythritol or buffer 2, and thus the procedure detailed in the Methods section was adopted. Organisms harvested and suspended in 327 mM-pentaerythritol gave reproducible rates of α -aminoisobutyrate transport when assayed over a 5 h storage period.

Kinetic studies of α -aminoisobutyrate transport

Studies on α -aminoisobutyrate transport as a function of α -aminoisobutyrate concentration demonstrated that a saturable component was involved (Fig. 1). Subsequent experiments gave a value for the K_m of the initial rate of α -aminoisobutyrate transport of 20 ± 8.5 mM ($n = 7$). Though not shown, when α -aminoisobutyrate transport was studied over the concentration range 25 μ M–50 mM the results obtained were consistent with the operation of a single transport system. The value of V_{max} , obtained from

Table 2. Specificity of α -aminoisobutyrate transport by *Crithidia fasciculata*

Transport was assayed aerobically in the presence of 10mM-glucose. Presumed competitors were added 10s before α -aminoisobutyrate (1 mM; 0.15 μ Ci/ μ mol) and the initial rate of transport was measured in duplicate from four samples removed in the 4min after initiation. The results are compiled from a series of experiments in which the control rates were 1–4 μ mol/min per g dry wt. Where applicable, amino acids were of the L-configuration.

Amino acid	Concentration (mM)	Relative rate of α -aminoisobutyrate transport (%)
None	—	100
Alanine	20	9
Serine	20	36
	100	17
Valine	20	37
	100	10
Threonine	20	81
	200	25
Tryptophan	20	17
Phenylalanine	20	36
	40	23
Methionine	20	53
	100	20
Glycine	20	25
	100	7
Proline	20	5
Histidine	20	74
	100	17
Cysteine	20	19
Aspartate	20	74
	100	49
Asparagine	20	95
Glutamate	20	96
	100	59
Glutamine	20	87
Isoleucine	20	50
	100	19
Lysine	20	63
	100	45
Arginine	20	44
	100	20

double-reciprocal plots, varied with different batches of the organism, falling within the range 20–90 μ mol/min per g dry wt. Specificity studies, in which presumed competitors were added 10s before α -aminoisobutyrate and the initial rate of α -aminoisobutyrate transport was then measured, showed that a system of broad specificity was operating (Table 2). With most of the amino acids that were competitors it was possible to demonstrate inhibition of more than 75%, the notable exceptions being lysine and aspartate. To assess whether any fraction of α -aminoisobutyrate transport was insensitive to these amino acids, experiments in which the initial rate of α -aminoisobutyrate transport was measured in the

Fig. 2. Uptake of α -aminoisobutyrate by *Crithidia fasciculata* under anaerobic conditions

Transport of α -aminoisobutyrate was measured as described in the Methods section in the pentaerythritol/buffer-2 system in the absence (●) and presence (○, 50mM; △, 10mM; □, 1mM) of glucose. The α -aminoisobutyrate concentration was 20mM.

presence of these inhibitors (concentration range 40–100mM) were performed. The results (not shown) were subsequently plotted as described by Inui & Christensen (1966) and demonstrated that essentially all the α -aminoisobutyrate transport at 1mM- α -aminoisobutyrate proceeded via a component that was subject to inhibition by these amino acids. As shown (Fig. 1), inhibition by phenylalanine was competitive, with K_i of 9mM. Similar findings were made with arginine (K_i 22mM) and alanine (K_i 2.4mM). The experiments so far described in this section were performed with the pentaerythritol/buffer 2 system. Measurement of the K_m for α -aminoisobutyrate uptake in buffer 1 yielded values in the range 16–17mM and values for V_{max} falling in the range 20–25 μ mol/min per g dry wt. Additionally in this buffer system, it was shown that proline and glycine were competitive inhibitors of α -aminoisobutyrate transport with values for the K_i of 0.8 and 6mM respectively.

Effect of inhibitors on α -aminoisobutyrate transport

The data presented in this paper are consistent with the operation of an active-transport system for α -aminoisobutyrate uptake by this organism. The major purpose of the experiments presented in this section was to investigate the energy-coupling mechanism for this system. To this end the sensitivity of α -aminoisobutyrate transport to a range of inhibitors has been studied under three different incubation conditions, namely under N_2 in the presence of hexoses (glucose and fructose), and aerobically in the

Table 3. *Effect of antimycin A or NaN₃ on α -aminoisobutyrate transport into Crithidia fasciculata under aerobic and anaerobic conditions*

Rates of α -aminoisobutyrate transport were measured in the pentaerythritol/buffer 2 system under the conditions indicated. The results are compiled from a series of experiments and the values in parentheses were the rates of α -aminoisobutyrate transport expressed as $\mu\text{mol}/\text{min}$ per g dry wt.

Assay conditions	Addition	Concentration	10mM-Hexose present	Relative initial rate of α -aminoisobutyrate transport (%)
Anaerobic	None		+	100 (0.24)
	Antimycin A	6.6 μM	+	100
		33 μM	+	100
Aerobic	None		—	100 (0.15)
	Antimycin A	3.3 μM	—	15
		6.6 μM	—	0
Aerobic	None		+	100 (0.28)
	Antimycin A	6.6 μM	+	97
		13.2 μM	+	74
		33 μM	+	78
Anaerobic	None		+	100 (0.31)
	NaN ₃	0.1 mM	+	89
		0.5 mM	+	20
		1.0 mM	+	9
Aerobic	None		—	100 (0.15)
	NaN ₃	0.1 mM	—	25
		0.5 mM	—	0
Aerobic	None		+	100 (0.38)
	NaN ₃	0.1 mM	+	94
		0.5 mM	+	83
		1.0 mM	+	46
		2.5 mM	+	0

presence and absence of hexoses. Experiments were conducted under anaerobic conditions for the reasons outlined in the introduction.

Transport of α -aminoisobutyrate was readily observed under anaerobic conditions (Fig. 2). Similar values for the initial rate of α -aminoisobutyrate transport were measured under aerobic or anaerobic conditions plus glucose or fructose. The major difference observed when the system was studied under anaerobic conditions was that α -aminoisobutyrate transport was almost wholly dependent upon added hexose (Fig. 2). This requirement could not be fulfilled by glucose 6- or 1-phosphate (10mM). As previously noted, appreciable transport of α -aminoisobutyrate occurred under aerobic conditions in the absence of added glucose or fructose. Measurement of the K_m and V_{max} for the initial rate of α -aminoisobutyrate transport, in three independent experiments under anaerobic conditions, gave values of 7, 9 and 9mM and 20, 31 and 23 $\mu\text{mol}/\text{min}$ per g dry wt. respectively. The substrate range employed was 90 μM –50mM.

As shown (Table 3), α -aminoisobutyrate transport under aerobic conditions was sensitive to antimycin A at concentrations that effectively inhibited respira-

tion (Table 8), provided that glucose and fructose were absent. Under anaerobic conditions, or aerobically in the presence of either sugar, α -aminoisobutyrate transport was insensitive to antimycin A. In contrast (Table 3), transport of α -aminoisobutyrate was sensitive to NaN₃ under all incubation conditions used. Respiration was only partially inhibited by azide at concentrations that completely inhibited α -aminoisobutyrate transport (Table 8). Suitable controls with NaCl demonstrated that the inhibition of transport was attributable to the N₃[−] anion. That this inhibition was reversible was shown in experiments in which the organism was incubated for 30min with NaN₃ or NaCl (both 2mM) under anaerobic conditions and subsequently washed and assayed for α -aminoisobutyrate transport under anaerobic conditions. Washing resulted in a restoration of α -aminoisobutyrate transport to the value observed in the washed NaCl control.

Experiments were performed with KCN to confirm the results obtained with antimycin A (Table 4). As reported by others (Edwards & Lloyd, 1973) and confirmed here (Table 8), respiration was completely inhibited by 1mM-KCN and this concentration was equally potent with regard to α -aminoisobutyrate

Table 4. Effect of KCN or an uncoupler on α -aminoisobutyrate transport into *Crithidia fasciculata* under aerobic and anaerobic conditionsThe conditions were as described in Table 3. Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Expt. no.	Assay conditions	Addition	Concentration	10mM-Hexose present	Relative initial rate of α -aminoisobutyrate transport (%)
1	Anaerobic	None	1.0mM	+	100 (0.56)
		KCN		+	82
2	Aerobic	None	0.25mM 0.5mM 1.0mM	—	100 (0.15)
		KCN		—	52
				—	31
				—	8
3	Aerobic	None	1.0mM	+	100 (0.5)
		KCN		+	79
4	Anaerobic	None	3 μ M 36 μ M 55 μ M	+	100 (0.65)
				+	79
		CCCP		+	36
				+	0
5	Aerobic	None	50 μ M	—	100 (0.42)
		CCCP		—	0
6	Aerobic	None	100 μ M 200 μ M 300 μ M	+	100 (0.21)
				+	70
		CCCP		+	7
				+	0

transport assayed under aerobic conditions (Expt. 2, Table 4). This sensitivity was only observed in the absence of added hexose since α -aminoisobutyrate transport became largely insensitive to this concentration of KCN when fructose or glucose was added (Expts. 1 and 3, Table 4). It should be noted that aerobically, in the presence of 1 mM-KCN, the addition of 10mM-glucose caused a 10-fold stimulation of α -aminoisobutyrate transport, though a similar addition did not stimulate respiration by KCN-inhibited organism (Expt. 3, Table 8).

The inhibitor studies so far presented were consistent with the ability of the organism to ferment glucose under anaerobic conditions or when respiration was inhibited. Under aerobic conditions, in the absence of glucose, the sensitivity of α -aminoisobutyrate transport to concentrations of KCN or antimycin A that inhibit respiration suggested that under these conditions little fermentative metabolism took place. Thus transport of α -aminoisobutyrate could be supported by an intermediate produced either by oxidative phosphorylation or fermentation, the most likely candidate being ATP. This was supported by the results of experiments with 2-deoxyglucose. α -Aminoisobutyrate transport was inhibited by more than 90% in the presence of 10mM-2-deoxyglucose under anaerobic, but not aerobic, incubation conditions. Although sodium arsenate (10mM, pH7.4) inhibited α -aminoisobutyrate transport by up to 55% under anaerobic conditions, such inhibition was

difficult to interpret since sodium phosphate (10mM, pH7.4) produced 40% inhibition.

α -Aminoisobutyrate transport was inhibited by *NN'*-dicyclohexylcarbodi-imide and *NN'*-di-isopropylcarbodi-imide, although the sensitivity was a function of the assay conditions (Table 5). In the presence of glucose, under aerobic or anaerobic conditions, higher concentrations of carbodi-imides were required to inhibit α -aminoisobutyrate transport compared with that required under aerobic conditions in the absence of glucose. These observations were consistent with at least two sites of carbodi-imide inhibition in this organism. Interaction of the more sensitive site was probably due to an inhibition of ATP production following the inactivation of the mitochondrial ATPase of this organism. This interpretation was supported by the observations that the lower concentrations of carbodi-imides used inhibited respiration in a manner that could be reversed by the addition of a suitable uncoupler (Expts. 5 and 6, Table 8). When an alternative means of generating ATP was available, such as glycolysis of added glucose, then higher concentrations of carbodi-imides were required to observe inhibition of α -aminoisobutyrate transport. This second site may be an ATPase located on the cytoplasmic membrane. This may also be the site of interaction of N_3^- under anaerobic conditions since this has been reported to be an inhibitor of such activity (e.g. Kobayashi *et al.*, 1977).

Table 5. *Effect of carbodi-imides on α -aminoisobutyrate transport into Crithidia fasciculata under aerobic and anaerobic conditions*

The conditions were as described in Table 3. Abbreviation: DCCD, *NN'*-dicyclohexylcarbodi-imide; DICD, *NN'*-di-isopropylcarbodi-imide.

Assay conditions	Addition	Concentration	10 mM-Glucose present	Relative initial rate of α -aminoisobutyrate transport (%)
Aerobic	None		—	100 (0.075)
	DCCD	1 μ M	—	72
		5 μ M	—	58
		10 μ M	—	14
Aerobic	None		+	100 (0.35)
	DCCD	10 μ M	+	86
		200 μ M	+	20
Anaerobic	None		+	100 (0.4)
	DCCD	75 μ M	+	38
		150 μ M	+	7
		300 μ M	+	0
Aerobic	None		—	100 (0.1)
	DICD	0.1 mM	—	72
		0.5 mM	—	26
		1.0 mM	—	0
Aerobic	None		+	100 (0.4)
	DICD	0.5 mM	+	87
		1.0 mM	+	64
		10 mM	+	3
Anaerobic	None		+	100 (0.66)
	DICD	1 mM	+	50
		5 mM	+	13

Table 6. *Effect of lipophilic cations on α -aminoisobutyrate transport into Crithidia fasciculata under aerobic and anaerobic conditions*

The conditions were as described in Table 3. Abbreviations: DDACl, dibenzyltrimethylammonium chloride; TPMPBr, methyltriphenylphosphonium bromide.

Expt. no.	Assay conditions	Addition	Concentration (mM)	10 mM-Glucose present	Relative initial rate of α -aminoisobutyrate transport (%)
1	Anaerobic	None		+	100 (0.66)
		DDACl	2	+	47
			5	+	27
			10	+	15
			20	+	8
			20	+	84
		NaCl	20	+	84
2	Anaerobic	None		+	100 (0.75)
		TPMPBr	0.5	+	66
			1.0	+	50
			5	+	22
			10	+	8
			10	+	102
		NaBr	10	+	102
3	Aerobic	None		—	100 (0.19)
		DDACl	10	—	11
		NaCl	10	—	111
		TPMPBr	10	—	0
		NaBr	10	—	75
4	Aerobic	None		+	100 (0.55)
		DDACl	10	+	9
		NaCl	10	+	94
		TPMPBr	10	+	0
		NaBr	10	+	78

The inhibitory effect of the carbodi-imides used in this study was shown to be time-dependent and irreversible for both sites. Thus the inhibition observed in the presence of low concentrations of carbodi-imides (20 μ M-*NN'*-dicyclohexylcarbodi-imide or 800 μ M-di-isopropylcarbodi-imide) under aerobic conditions in the absence of glucose was not reversed when the organism was subsequently washed. Organism treated in this manner retained appreciable α -aminoisobutyrate-transport activity when glucose was added. Incubation with higher concentrations of carbodi-imides (300 μ M-*NN'*-dicyclohexylcarbodi-imide or 2 mM-*NN'*-di-isopropylcarbodi-imide) resulted in severe inhibition of α -aminoisobutyrate transport that was not relieved by subsequent washing or addition of glucose.

α -Aminoisobutyrate transport was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone under anaerobic conditions (Expt. 4, Table 4). Similar concentrations also inhibited aerobically supported α -aminoisobutyrate transport, although somewhat higher concentrations were required in the presence of glucose (Expts. 5 and 6, Table 4). Although only the data for carbonyl cyanide *m*-chlorophenylhydrazone are presented, essentially similar findings were made with other uncouplers, e.g. 58 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or 2 mM-2,4-dinitrophenol caused greater than 90% inhibition of α -aminoisobutyrate transport under anaerobic conditions.

The lipophilic cations dibenzyltrimethylammonium and methyltriphenylphosphonium also inhibited α -aminoisobutyrate transport when this was assayed under anaerobic conditions (Expts. 1 and 2, Table 6), and a similar sensitivity was shown under aerobic conditions (Expts. 3 and 4, Table 6). As shown, suitable controls established that the inhibition observed was not due to Cl^- or Br^- .

α -Aminoisobutyrate transport was inhibited by HgCl_2 and *N*-ethylmaleimide under all three assay conditions employed (Table 7), indicating a role for components with SH groups in the overall process.

Chlorhexidine and 4-chloro-7-nitrobenzofuran, which have been reported as ATPase inhibitors in other systems (Harold *et al.*, 1969; Ferguson *et al.*, 1976), were found to inhibit α -aminoisobutyrate transport (Table 7). No major difference in sensitivity to these inhibitors was noted when aerobic incubation conditions were used. In experiments similar to those described for the carbodi-imides, the effect of these inhibitors was not reversed by washing.

α -Aminoisobutyrate transport, assayed under anaerobic conditions, was insensitive to phlorrhizin (10 mM), quercetin (295 μ M) and oligomycin (100 μ g/ml). α -Aminoisobutyrate transport, under aerobic conditions in the absence of glucose, was also insensitive to this concentration of oligomycin.

Table 7. Effect of mercuric chloride, *N*-ethylmaleimide, 4-chloro-7-nitrobenzofuran or chlorhexidine on α -aminoisobutyrate transport into *Crithidia fasciculata* under anaerobic conditions

The conditions were as described in Table 3. All assays were performed under anaerobic conditions in the presence of 10 mM-glucose. Abbreviations: NEM, *N*-ethylmaleimide; NBDCl, 4-chloro-7-nitrobenzofuran chloride.

Addition	Concentration	Relative initial rate of α -aminoisobutyrate transport (%)
None		100 (0.37)
HgCl_2	1 μ M	91
	10 μ M	42
	50 μ M	3
None		100 (0.3)
NEM	0.2 mM	13
	1 mM	3
	5 mM	0
None		100 (0.16)
NBDCl	1 μ M	100
	10 μ M	74
	30 μ M	59
	50 μ M	7
	100 μ M	0
None		100 (0.41)
Chlorhexidine	5 μ g/ml	76
	25 μ g/ml	12
	50 μ g/ml	0

Studies on respiration

With freshly harvested organisms subsequently stored at 4°C before assay, respiration was only marginally stimulated by addition of glucose or fructose. The interaction of respiration with various compounds was studied under the same conditions of assay as those for α -aminoisobutyrate transport. Such studies were undertaken to confirm the previous interpretation concerning the energy source for α -aminoisobutyrate transport and the role of glucose in the organism when respiration was inhibited. Thus KCN and antimycin A severely inhibited respiration (Expts. 1 and 2, Table 8) at similar concentrations to these required to inhibit α -aminoisobutyrate transport under aerobic conditions in the absence of hexose. The addition of glucose to such inhibited organisms did not stimulate respiration, although, as shown previously (Tables 3 and 4), α -aminoisobutyrate transport was markedly stimulated.

Noticeably higher concentrations of NaN_3 were required to inhibit respiration than to inhibit α -aminoisobutyrate transport under any of the incubation conditions studied (Expt. 3, Table 8). The addition of 10 mM-glucose to organisms whose respiration was 85% inhibited by 37 mM- NaN_3 did not stimulate respiration.

Table 8. *Effect of transport inhibitors on respiration by Crithidia fasciculata*

The rate of respiration was measured as described in the Methods section. The values in parentheses are the absolute rates of O₂ consumption expressed as $\mu\text{mol/min per g dry wt.}$ Abbreviations are as in the other Tables.

Experiment no.	Addition	Concentration	Relative rate of respiration (%)
1	None		100 (19.6)
	Antimycin A	2 μM	21
		4 μM	19
		6 μM	14
	Antimycin A Plus glucose	10 mM	12
2	None		100 (25.2)
	KCN	0.33 mM	20
		0.67 mM	12
		1.33 mM	0
		1.33 mM	0
	KCN Plus glucose	10 mM	0
	None		100 (28.6)
3	NaN ₃	0.5 mM	100
		1.0 mM	85
		2.0 mM	68
		3.0 mM	51
		12 mM	32
4	None		100 (59.2)
	CCCP	20 μM	166
		40 μM	170
		50 μM	166
		60 μM	148
5	None		100 (62.4)
	DCCD	300 μM	4
	DCCD	300 μM	4
	Plus CCCP	40 μM	4
6	None		100 (47.8)
	CCCP	40 μM	169
	DCCD	20 μM	25
	DCCD	20 μM	25
	Plus CCCP	40 μM	168
7	None		100 (50.4)
	DCCD	20 μM	21
	DCCD	20 μM	30
	Plus glucose	10 mM	
	DCCD	20 μM	
	Plus glucose Plus CCCP	10 mM 40 μM	135

Although only the data for carbonyl cyanide *m*-chlorophenylhydrazine are presented (Expt. 4, Table 8), 2,4-dinitrophenol and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine also acted as uncouplers in this organism, 31.5 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and 1.25 mM-

Table 9. *Effect of inhibitors of α -aminoisobutyrate transport on glycolysis by Crithidia fasciculata*

Glycolysis was measured as acid production from glucose in a pH-stat as described in the Methods section. The values from two independent series of experiments are presented. The uninhibited rate for these experiments was in the range 17–35 $\mu\text{g-ions of H}^+/\text{min per g dry wt.}$ Abbreviations are as in the other Tables.

Addition	Concentration	Relative rate of glycolysis (%)	
		Expt. 1	Expt. 2
None		100	100
HgCl ₂	50 μM	100	86
NBDCI	50 μM	53	54
DICD	10 mM	79	92
DCCD	300 μM	50	59
NaN ₃	2 mM	94	93
2-Deoxyglucose	10 mM	25	21
NEM	5 mM	0	0
Chlorhexidine	50 $\mu\text{g/ml}$	43	44
2,4-Dinitrophenol	2 mM	60	58
CCCP	55 μM	34	32
FCCP	60 μM	0	0
TPMPBr	10 mM	103	112
DDACI	20 mM	108	115

2,4-dinitrophenol stimulating respiration 1.7-fold and 1.3-fold respectively. High concentrations of *NN'*-dicyclohexylcarbodi-imide (300 μM) caused an inhibition of respiration that could not be reversed by the addition of uncoupler (Expt. 5, Table 8), whereas the inhibition observed with 20 μM -*NN'*-dicyclohexylcarbodi-imide was fully reversed by such addition (Expt. 7, Table 8). The addition of 10 mM-glucose did not reverse the inhibition of respiration by 20 μM -*NN'*-dicyclohexylcarbodi-imide (Expt. 7, Table 8), although α -aminoisobutyrate transport was markedly stimulated under those conditions. Similar findings to these made with *NN'*-dicyclohexylcarbodi-imide were made with *NN'*-diisopropylcarbodi-imide, concentrations above 2.5 mM causing an inhibition of respiration not reversed by addition of uncoupler, whereas the inhibition caused by 0.8 mM-*NN'*-diisopropylcarbodi-imide was fully relieved by the addition of carbonyl cyanide *m*-chlorophenylhydrazine.

Respiration was insensitive to oligomycin (100 $\mu\text{g/ml}$).

Metabolic effects of inhibitors

The effect of the major inhibitors of α -aminoisobutyrate transport on glycolysis, as measured by acid production in a pH-stat, was studied under similar conditions to the transport measurements, except

that the buffering capacity was lowered and antimycin A was added to inhibit respiration (Table 9). The concentrations of inhibitors employed were those that produced greater than 90% inhibition of α -aminoisobutyrate transport assayed under anaerobic conditions. These experiments were undertaken to assess the sensitivity of glucose transport to the inhibitors since, as α -aminoisobutyrate transport under anaerobic conditions was glucose-dependent, their primary effect could be on glucose transport. In addition such experiments yield information as to their specificity of action.

Discussion

These results show that *C. fasciculata* was capable of maintaining large concentration gradients of α -aminoisobutyrate across its cytoplasmic membrane. Kinetic studies were consistent with the operation of a single system, of broad specificity, though, as noted by Christensen (1975), it is not possible to exclude the operation of more than a single system by purely kinetic procedures. Further attempts to analyse the homogeneity of α -aminoisobutyrate transport by using the approaches described by Inui & Christensen (1966), namely the 'constant ratio test', have been unsuccessful. This test requires the use of a competing amino acid that is known to enter via a single system. To establish this the transport of glycine and proline was investigated, but in both cases non-linear double-reciprocal plots were obtained (J. P. Robinson & M. Midgley, unpublished observations), suggesting the involvement of more than a single component in transport of these amino acids. Thus it is not suggested that this organism has a single system for entry of all amino acids, rather that a number of systems operate, and, so far as the approaches adopted allow, one of these was involved in α -aminoisobutyrate uptake.

Transport of α -aminoisobutyrate showed no marked requirement for inorganic ions such as K^+ or Na^+ , but did demonstrate a relatively non-specific osmotic requirement. This insensitivity to these ions (and ouabain) suggested that co-transport or symport with these species was not occurring. The possibility remained that a proton-symport mechanism was operating and evidence for this came from the sensitivity of α -aminoisobutyrate transport to various inhibitors.

The effect of KCN, antimycin A and 2-deoxyglucose under different incubation conditions (Tables 3 and 4) was consistent with α -aminoisobutyrate transport being supported by ATP, produced by oxidative phosphorylation or glycolysis. The sensitivity of α -aminoisobutyrate transport, under anaerobic conditions, to uncouplers, lipophilic cations and various presumed ATPase inhibitors was con-

sistent with the operation of a secondary active-transport system for α -aminoisobutyrate driven by an electrochemical gradient of protons maintained by a proton-translocating ATPase. Thus by analogy with other systems (Harold, 1972; Hamilton, 1975) it is suggested that these compounds prevent the generation or maintenance of an electrochemical gradient of protons across the cytoplasmic membrane. It is appreciated that an approach that relies exclusively upon the use of inhibitors is by no means definitive since the specificity of many of these compounds is not clearly established. For example, the sensitivity of α -aminoisobutyrate transport to chlorhexidine was probably due to its interaction with the cell membrane, resulting in a disruption of the permeability barrier, since, as noted by Harold (1970), as well as being an ATPase inhibitor it also acts as a cationic detergent. This explanation was supported by the observation that, if chlorhexidine was added to organism that had accumulated α -aminoisobutyrate, rapid efflux was observed. In contrast when *NN'*-dicyclohexylcarbodi-imide, *NN'*-di-isopropylcarbodi-imide, $HgCl_2$ or NaN_3 was added there was no loss of α -aminoisobutyrate (M. Midgley, unpublished observations). The inhibition of α -aminoisobutyrate transport by $HgCl_2$ may reflect the involvement of an SH group in the overall process; similarly the inhibition by 4-chloro-7-nitrobenzofuran chloride may imply no more than this. The site of interaction was probably not triose phosphate dehydrogenase, the likely site of *N*-ethylmaleimide inhibition, since glycolysis was not inhibited by $HgCl_2$ and only partially inhibited by 4-chloro-7-nitrobenzofuran chloride.

Thus these compounds do not inhibit ATP generation but act at some site concerned with transport after this. Although carbodi-imides are widely used to study ATPases their specificity has not been widely established. However, *NN'*-dicyclohexylcarbodi-imide and *NN'*-di-isopropylcarbodi-imide inhibited glycolysis by 46% and 15% respectively, thus making it unlikely that ATP generation was their site of action. An example of the specificity of *NN'*-dicyclohexylcarbodi-imide comes from work in this laboratory concerning the transport of α -aminoisobutyrate into *Pseudomonas fluorescens*, a strictly aerobic organism. This can be treated with *NN'*-dicyclohexylcarbodi-imide (800 μM) under conditions leading to more than 90% inactivation of the ATPase associated with its cell membrane, with no impairment of α -aminoisobutyrate transport, which, in this organism, is supported by electron transport (M. C. Stephenson, M. Midgley & E. A. Dawes, unpublished work). The concentration of *NN'*-dicyclohexylcarbodi-imide (300 μM) required to inhibit α -aminoisobutyrate transport under anaerobic conditions was rather higher than that required to inhibit the mitochondrial ATPase of this organism. Similar

concentrations of carbodi-imides have been shown to inhibit ATPases from other organisms, e.g. the ATPase of *Chlorobium thiosulfatophilum* was inhibited by 10mM-*NN'*-di-isopropylcarbodi-imide or 300 μ M-*NN'*-dicyclohexylcarbodi-imide (Burns & Midgley, 1976). Reservations may also be applied when interpreting the action of the uncouplers used in this study. It seems unlikely that their sole action was to produce a proton-permeable cytoplasmic membrane, thereby collapsing any electrochemical gradient of protons present, especially since glycolysis was inhibited to different extents by the different uncouplers employed (Table 8). NaN_3 , dibenzyl-dimethylammonium and methyltriphenylphosphonium cations produced no inhibition of glycolysis yet severely inhibited α -aminoisobutyrate transport, suggesting that these compounds interfered in some way with the coupling of these processes. While fully acknowledging these drawbacks concerning the specificity of the inhibitors used in this study the characteristics of α -aminoisobutyrate transport observed here suggest that the mechanism proposed is a reasonable working model on which to base more positive approaches. Attempts to detect α -aminoisobutyrate-induced proton movements in experiments of the type described by West & Mitchell (1973) for the lactose-transport system in *Escherichia coli* have been unsuccessful (M. Midgley, unpublished observations). Similar findings have been reported for *Trypanosoma brucei* and threonine transport (Voorheis, 1977). At this stage in the investigation of the energy-coupling mechanism it is perhaps premature to rule out proton symport exclusively on this basis, because of the probably steady-state nature of the maintenance of an electrochemical gradient of protons across the cytoplasmic membrane. Two major approaches appear profitable, namely to see if such a gradient does in fact exist and to isolate the cytoplasmic membrane and look for an ATPase sensitive to the compounds inhibiting α -aminoisobutyrate transport associated with this.

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