Active transport of L-proline in the protozoan parasite *Trypanosoma brucei brucei*

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The characteristics of L-proline transport in the procyclic form of *Trypanosoma brucei* were studied by using L-[¹⁴C]proline and a quick separation technique by centrifugation through an oil mixture. L-Proline uptake displayed typical Michaelis–Menten kinetics, with a K_m of 19 μ M and a maximum transport velocity of 17 nmol/min per 10^8 cells at 27 °C. The maximum concentration gradient factor obtained after 1 min of incubation was 270-fold in 0.02 mM proline. Cells permeabilized with 80 μ M digitonin were still able to accumulate ¹⁴C label, but to a lower extent. The temperature-dependence of proline uptake gave an apparent activation energy of 74.9 kJ·mol⁻¹. In competition studies with a 10-fold excess of structural analogues, L-alanine, L-cysteine and L-azetidine-2-carboxylate were found to inhibit L-proline uptake. Variation of pH or addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone ('CCCP') did not

affect proline transport, showing that it is not driven by a protonmotive force. The absence of Na⁺, with or without monensin, did not affect proline transport. The absence of K⁺ and the addition of the Na⁺,K⁺-ATPase inhibitor ouabain had no significant effect on proline uptake activity. The thiol-modifying reagent iodoacetate (10 mM) decreased proline uptake by half. KCN (1 mM) inhibited proline uptake to a lesser extent, and the degree of inhibition was proportional to the intracellular ATP concentration. Preliminary experiments on proline transport in plasma-membrane vesicles of the cells, using a filtration technique, showed an uptake of proline (0.67 nmol/mg of protein) by the vesicles, but only in the presence of intravesicular ATP. The results thus obtained suggest that the proline carrier system in *T. brucei* is ATP-driven and independent of Na⁺, K⁺ or H⁺ co-transport.

INTRODUCTION

The protozoan parasite *Trypanosoma brucei*, the agent of human sleeping sickness and nagana in domestic animals, has been extensively studied for its variant surface glycoprotein coat and its glucose metabolism. Knowledge of the membrane physiology of the parasite in either form (bloodstream and procyclic stages) is also essential in order to find effective drugs against these diseases.

Few studies have characterized the mode of amino acid uptake from the environment by *Trypanosoma brucei*. *T. brucei* utilizes L-proline as its main source of energy and carbon when it is in the procyclic or insect stage (Evans and Brown, 1972), but the energy source of the bloodstream form is glucose. Some other species of Trypanosomatidae family can compensate for the absence of carbohydrates from the growth medium by transporting and oxidizing proline. For instance, in *Leishmania donovani*, proline transport was found to be coupled to proton translocation (Bonay and Cohen, 1983; Zilberstein and Dwyer, 1985; Glaser and Mikkada, 1992).

The uptake of various amino acids in some *Trypanosoma* species has been examined, showing a mediated transport mechanism rather than a passive diffusion system for most amino acids studied (Southwork and Read, 1972; Ruff and Read, 1974; Hansen, 1979), but the transport systems were never fully characterized.

In eukaryotes, amino acids traverse biological membranes by mediated diffusion and a multiplicity of carrier systems classified as Na⁺-dependent and Na⁺-independent (Christensen, 1984). Since little is known about how the procyclic form of *T. brucei* transports proline, its main source of carbon, we investigated the mechanism of this imino acid uptake into the cells: its specificity,

kinetics and driving force. The present study shows that a concentrative and active transport exists, but is Na⁺-independent and not coupled to proton translocation, as found in *Leishmania*.

MATERIALS AND METHODS

Procyclic forms of *Trypanosoma brucei brucei* (strain STIB 366) were grown as described by Brun and Schonenberger (1979) in SDM-79 medium. After 3 days of growth, cells were washed twice in PBS, pH 7.6 (50 mM Na₂HPO₄, 3.7 mM NaH₂PO₄, 66 mM NaCl) supplemented with 56 mM glucose and 20 mM KCl (PSGK buffer) and then resuspended in about 2 ml of the same buffer. The cell suspension was kept on ice during the experiment. Cell density [(2–5) × 10⁸ cells/ml] was determined by counting in a Petroff–Hauser cell. Bloodstream forms of *T. brucei* (strain STIB 345AB) were grown on fibroblasts (*Microtus montana* embryo fibroblasts) in a culture medium as described by Baltz et al. (1985).

Uptake of proline was initiated by injection of 60 μ l of cell suspension into 180 μ l of PSGK buffer in the presence of L-[¹⁴C]proline (3.5–35 mCi/mmol) preincubated at 27 °C. The uptake experiment, performed at 27 °C unless otherwise stated, was interrupted after the required incubation time by centrifugation for 10 s in a Microfuge B (Beckman) of a 180 μ l sample through 200 μ l of oil mixture (diethyl phthalate/paraffin oil, 9:1, v/v). All measurements were done in at least duplicate. The cells were intact and fully motile after centrifugation. The respective radioactivities of the cell pellet and the supernatant were determined by liquid-scintillation counting. Extracellular and water-accessible volumes were measured by using [¹⁴C]poly-(ethylene glycol) and ³H₂O respectively; the pellet radioactivity

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; PSGK, PBS/glucose/K⁺ buffer.

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was corrected accordingly for [^{14}C]proline in the interstitial volume.

When inhibitors or ionophores were used, they were first dissolved in ethanol (valinomycin, monensin), dioxan [ouabain, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)] or PSGK buffer (sodium iodoacetate, KCN), then added to the cell suspension at the desired concentration and preincubated for 1–10 min on ice, before an uptake experiment. Control samples were prepared with the same amount of solvent in order to take their possible effect into account.

Incorporation of proline into proteins was measured by trichloroacetic acid extraction as follows. After incubation of the cells in the presence of [^{14}C]proline, the cells were centrifuged through a layer of 160 μl of oil mixture and collected in a 20 μl bottom layer of 18 % (w/v) trichloroacetic acid and 17 % (w/v) sucrose. Four identical pellets of cells were pooled and then extracted in 1 ml of 20 % trichloroacetic acid for 30 min on ice. The precipitated protein fraction was then separated from the soluble fraction by centrifugation of the diluted extract through the oil mixture.

Amino acids were extracted after incubation of the cells in the presence of [^{14}C]proline for different lengths of time. Pellets of cells were centrifuged through oil and collected in a bottom layer of 40 % trichloroacetic acid. Pellets were pooled and resuspended in 200 μl of distilled water. Traces of oil were extracted with diethyl ether, and the aqueous phase was dried by evaporation with a Speedvac apparatus. The extracts were dissolved in 20 % ethanol, placed on a silica-gel t.l.c. plate (0.25 mm $\times$ 20 cm $\times$ 20 cm) and separated with a phenol/water (3:1, w/v) solvent. Unlabelled proline, alanine and glutamic acid were used as controls. The plate was scanned on an automatic t.l.c.-linear analyser (Tracemaster 20, Berthold). Amino acids were then revealed with a ninhydrin solution (Johnson and Strecker, 1962).

Cellular ATP was extracted as described by Zilberstein and Dwyer (1985). ATP concentrations were measured by mixing 25 μl of cellular extract in glycylglycine buffer with 25 μl of firefly lantern extract (10 mg/ml) in a scintillation vial. Then 10 s after mixing, the chemiluminescence was measured for 30 s in a Beckman LS9000 scintillation counter set to maximum sensitivity. Background luminescence without ATP was 20–30 c.p.m. and, from the ATP calibration curve, 150 000 c.p.m. were recorded per 25 pmol of ATP.

Proline transport experiments were performed on membrane vesicles from *T. brucei*. The membrane purification procedure was adapted from Zingales et al. (1979) and Rovis and Baekkeskov (1980) as follows. The cellular pellet (about 10^{10} cells) was washed three times in PSGK buffer, resuspended in 20 ml of 10 mM Tris/HCl/10 mM MgCl_2 buffer, pH 7.5, and sonicated for 5 min (15 s bursts) in a MSE sonicator. The homogenate was diluted to 40 ml with the same buffer and centrifuged at 750 g (15 min, 4 $^{\circ}\text{C}$); the pellet of intact cells and nuclei was discarded and the supernatant was centrifuged at 5000 g (30 min, 4 $^{\circ}\text{C}$) to remove microbodies (Rovis and Baekkeskov, 1980). The microsomal fraction was then pelleted at 14000 g (1 h, 4 $^{\circ}\text{C}$), resuspended in the same buffer and re-pelleted in the same conditions. The washed microsomal pellet was resuspended in 1 ml of buffer, homogenized and loaded on a discontinuous sucrose gradient (55, 40, 35, 30, 12 % sucrose in 10 mM Tris/HCl, pH 7.5). The gradient was centrifuged for 3 h at 26000 g , 4 $^{\circ}\text{C}$. The partially purified plasma-membrane fraction was collected at the 35/40 %-sucrose interface, diluted 5-fold in 10 mM Tris/HCl/2.0 M sucrose, pH 7.5, centrifuged at 100000 g (45 min, 4 $^{\circ}\text{C}$) and resuspended in 1 ml of the same Tris/sucrose buffer to obtain 0.6–1 mg of protein/ml. ATP (10 mM final concn.) was added to a sample of the vesicle

suspension and equilibrated by two cycles of freeze-thawing. Control vesicles were treated similarly but without ATP. Transport was initiated by addition of 75 μl of ice-cold vesicle suspension to 75 μl of Tris/sucrose buffer containing [^{14}C]proline (0.13 mM, 25 mCi/mmol), 7 mM NaCl and 7 mM KCl, and preincubated at 38 $^{\circ}\text{C}$. Uptake was interrupted after 20 s by filtration of a 130 μl sample on a 0.45 μm -pore nitrocellulose filter. The filter was immediately rinsed with 4 ml of ice-cold Tris/sucrose buffer. The filter radioactivity was measured by liquid-scintillation counting after thorough vortex-mixing. Blanks were performed by using a very short incubation time (5 s) for the vesicles in the presence of [^{14}C]proline with a large excess (120-fold) of unlabelled proline.

Protein concentrations were measured as described by Lowry et al. (1951), with BSA as standard.

Results are expressed as mean values of at least two replicates, and experimental errors are given as S.D. Significance was tested with a paired Student's *t* test.

Sodium iodoacetate, ouabain, monensin, digitonin, valero-lactam and 5-aminopentanoic acid were purchased from Fluka; valinomycin, L-azetidine-2-carboxylic acid and firefly lantern extract were from Sigma; CCCP was from Calbiochem; L-[^{14}C]proline (250 mCi/mmol) was from Amersham International; and the nitrocellulose filters were from Sartorius.

RESULTS

Kinetic profile of proline transport

The time-course experiment of proline uptake in *T. brucei* cells was performed at 27 $^{\circ}\text{C}$ with an initial proline concentration of 1 mM. The curve describing the internal [^{14}C]proline content as a function of time (Figure 1) shows an initial linear phase for the first 2 min of incubation, followed by a curve tending towards a steady state (170 nmol/ 10^8 cells in 8 min and 280 nmol/ 10^8 cells in 20 min of uptake). Consequently, most uptake experiments were then performed during the linear uptake phase, usually 35 s. All pellet radioactivities were corrected for ^{14}C label in the extracellular fluid, which can be found to be 3.1 ± 0.2 $\mu\text{l}/10^8$ cells, i.e. 63 % of the total pellet volume (result of four independent determinations).

The concentration-dependence of L-proline uptake for a 35 s incubation (Figure 2) follows a Michaelis-Menten kinetic scheme ($K_m = 18.7 \pm 1.7$ μM , $V_{\text{max}} = 16.1 \pm 0.5$ nmol/min per 10^8 cells; results of two independent experiments). A similar experiment using cells permeabilized with 80 μM digitonin showed a much lower uptake of proline, which increased nearly proportionally to the external concentration of proline. This result suggests a high intracellular concentration of proline, either as free solutes or perhaps partially incorporated into proteins.

Figure 1 Uptake of L-proline by *T. brucei* cells as a function of time

Initial concentration of proline, 1 mM; incubation temperature, 27 $^{\circ}\text{C}$.

Figure 2 Concentration-dependence of L-proline uptake

Cells were incubated at 27 °C for 35 s. ■, Control experiment with intact cells ($n = 4$); □, cells permeabilized with 80 μ M digitonin ($n = 2$).

In order to determine whether the accumulation of [14 C]proline in the cells was due to its incorporation into proteins, cell proteins were extracted in trichloroacetic acid after a 35 s or 30 min incubation in the presence of [14 C]proline. The radioactivity found in the trichloroacetic acid-non-soluble fraction was below detectability after 35 s and less than 0.2 % of the total radioactivity after 30 min. These results show that only a negligible fraction of the transported proline is incorporated into proteins after 30 min of incubation.

T.l.c. analysis of intracellular amino acids showed that after a 35 s proline uptake nearly all the radioactivity was found in proline ($97.5 \pm 1.7\%$), with a very weak peak (about 2.5 %) corresponding to glutamic acid. After 5 min and 10 min of proline uptake, the fractions of labelled proline were lower ($86.2 \pm 1.0\%$ and $85.1 \pm 0.9\%$ respectively) and the rest of the radiolabel was found in glutamic acid. No traces of radiolabelled alanine were detected in the cell extracts. Consequently, all the radioactivity measured inside the cells after a short incubation period (35 s to some minutes) corresponds to the free solute.

Assuming that proline is homogeneously distributed in the intracellular space, and considering an internal accessible volume of $1.8 \pm 0.3 \mu\text{l}/10^8$ cells (as determined by subtraction of the [14 C]poly(ethylene glycol) accessible volume from the $^3\text{H}_2\text{O}$ -accessible volume), the intracellular concentration of proline can be estimated. After 1 min of uptake, internal concentrations were 7.9–14.7 mM for an external concentration of 0.1 mM proline, depending on experiments. Thus a range of concentration gradients of 80–150-fold was obtained after 1 min of incubation in 0.1 mM proline. In presence of 0.02 mM external proline, the concentration gradients ranged from about 180- to 270-fold. In the presence of digitonin, the accumulation of [14 C]proline was on average 3.4 times lower than for intact cells. This result suggests that, despite a permeabilized cytoplasmic membrane (Kiari and Njogu, 1983), the cell can still accumulate some proline or its metabolites. Therefore, proline uptake in *T. brucei* cells is carried out against its concentration gradient and requires an active transport system.

A similar transport experiment was performed on the bloodstream form of *T. brucei*. A maximum concentration gradient of 18-fold was obtained after 1 min of incubation at 27 °C with an external proline concentration of 0.02 mM, showing that the bloodstream form can also concentrate proline to some extent, but much less than the procyclic form.

In order to calculate the activation energy (E_a) for proline uptake, experiments with an initial L-proline concentration of

Table 1 Effect of 1 mM amino acids or analogues on the uptake of 0.1 mM proline by *T. brucei*

Inhibitor	Inhibition (% , + S.D.)
None	0 ± 4.5
Unlabelled proline	93.3 ± 1.1
D-Proline	16.7 ± 0.6
L-Azetidine-2-carboxylic acid	46.1 ± 0.2
Hydroxyproline	65.8 ± 1.0
L-Alanine	51.1 ± 1.8
L-Cysteine	35.1 ± 3.8
L-Threonine	13.3 ± 3.2
L-Serine	12.9 ± 0.3
L-Tyrosine	1.6 ± 0.5
L-Lysine	3.9 ± 2.9
L-Arginine	3.0 ± 3.8
L-Methionine	4.1 ± 3.2
L-Valine	12.7 ± 2.4
L-Tryptophan	8.2 ± 3.1
L-Histidine	7.0 ± 5.4
L-Glutamic acid	2.7 ± 2.4
DL-Pyrogutamic acid	3.6 ± 4.3
L-Glutamine	5.5 ± 3.6
DL-Asparagine	9.9 ± 1.0
L-Phenylalanine	9.7 ± 2.6
L-Leucine	7.3 ± 1.6
L-Isoleucine	5.2 ± 0.6
L-Glycine	9.3 ± 3.8
L-Citrulline	8.0 ± 1.4
L-Ornithine	2.7 ± 1.0
Putrescine	-3.4 ± 2.1
Cadaverine	1.0 ± 2.8

0.05 mM were carried out at nine different temperatures in the range 6–28 °C. Decreasing the temperature lowered the rate of proline uptake considerably (results not shown). Uptake values corrected for external proline were used to plot $\ln v$ against $1/RT$ according to the Arrhenius equation, with R the universal gas constant ($8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), T the absolute temperature and v the proline uptake velocity (in nmol/min per 10^8 cells). The negative slope calculated by linear regression analysis gave an apparent E_a of $74.9 \text{ kJ} \cdot \text{mol}^{-1}$, with a regression coefficient of 0.9969.

Effect of structural analogues

The uptake of [14 C]proline was measured in the presence of a 10-fold higher concentration of other unlabelled amino acids or structural analogues of L-proline. Of the 20 amino acids tested, only the zwitterionic amino acids L-alanine and L-cysteine decreased significantly the uptake of proline (Table 1). The other amino acids tested did not induce a significant inhibition of proline uptake.

As expected, the structural analogues L-hydroxyproline and L-azetidine-2-carboxylate were potent inhibitors. The stereoisomer D-proline only slightly inhibited L-proline uptake, showing a moderate stereospecificity of the carrier.

Transport driving force

In *Leishmania donovani* promastigotes, the transport of L-proline is driven by a protonmotive force across the membrane (Zilberstein and Dwyer, 1985). In order to determine whether it is also the case in *Trypanosoma*, the influence of pH was first investigated. No significant change in the proline uptake rate was

Table 2 Effect of ouabain, CCCP, sodium iodoacetate and KCN on L-proline uptake by *T. brucei*

Results are expressed as means $\pm$ S.D. of replicates. 'Incubation' refers to incubation of the cell suspension in presence of 0.5 mM proline.

Inhibitor	Preincubation (min)	Incubation	Uptake (% of control)
Ouabain (50 μ M)	20	35 s	102.7 $\pm$ 4.8
CCCP (10 μ M)	1	35 s	100.9 $\pm$ 1.0
LiCl (no Na ⁺)		35 s	91.5 $\pm$ 3.6
Monensin (2 μ M)	3	35 s	101.2 $\pm$ 1.1
No K ⁺		35 s	93.9 $\pm$ 2.6
125 mM KCl		35 s	104.4 $\pm$ 3.8
Valinomycin (1 μ M)	7	35 s	103.3 $\pm$ 4.7
Iodoacetate (1 mM)	8	35 s	106.3 $\pm$ 2.7
Iodoacetate (10 mM)	2	5 min	55.7 $\pm$ 0.3
Iodoacetate (10 mM)	2	10 min	54.1 $\pm$ 1.4
KCN (1 mM)	2	1 min	95.3 $\pm$ 0.9
KCN (1 mM)	2	5 min	76.9 $\pm$ 6.0
KCN (1 mM)	2	10 min	74.2 $\pm$ 2.8

observed when the cells were incubated at the control pH, i.e. pH 7.6 (16.30 ± 0.14 nmol/min per 10^8 cells), or at pH 6.9 (16.14 ± 1.00) and 8.0 (16.04 ± 0.30) in 150 μ M proline ($P < 0.01$). Moreover, the protonophore CCCP had no effect at a concentration of 10 μ M on 35 s uptake experiments after a 1 min preincubation with the inhibitor (Table 2). These results indicate that *T. brucei* does not use an electrochemical proton gradient as energy source to translocate proline.

Proline uptake was measured in the presence and absence of Na⁺ in order to determine whether it is a Na⁺-dependent transport. In these experiments, NaCl was replaced by LiCl (Nakanishi et al., 1989). The presence of Li⁺ seemed to induce a slight inhibition ($8.5 \pm 3.6\%$) of proline uptake, but the Na⁺ ionophore monensin (2 μ M) had no effect on the transport of proline with or without Na⁺ (Table 2). The K⁺-dependence of proline transport was also investigated. No significant inhibition of proline uptake was measured in the absence of K⁺, and the ionophore valinomycin (1 μ M) did not affect proline uptake (Table 2). Moreover, addition of the Na⁺,K⁺-ATPase inhibitor ouabain (50 μ M; 20 min preincubation in the dark) had no effect on the imino acid transport by *T. brucei* (Table 2).

Metabolic inhibitors were used in order to establish a correlation between proline uptake and ATP concentration. Since a low concentration of iodoacetate (1 mM) had no effect on proline uptake, higher concentrations had to be used (Table 2). The cell suspension was divided into three batches; a control suspension and two suspensions with 10 mM iodoacetate and 1 mM KCl respectively. Intracellular ATP concentrations were measured in the cell suspension before addition of inhibitor and also after 5 and 10 min of proline uptake by samples of the three cell suspensions. Proline uptake was measured in parallel for the same suspensions.

The results of the control samples show a marked increase in ATP concentration after 5 and 10 min of proline uptake.

Sodium iodoacetate, a thiol-modifying reagent, decreased proline uptake by about half of the control value after 5 and 10 min of incubation in the presence of proline (Table 2). In the same cell suspension, the ATP concentration first increased after 5 min of uptake, but less than in the control sample, followed by a diminution of ATP concentration after 10 min in the presence of proline (Table 3).

Table 3 Effect of iodoacetate (10 mM) and KCN (1 mM) on intracellular ATP content

Results are expressed in nmol/ 10^8 cells ($\pm$ S.D.) measured before proline uptake (Initial), after 5 min of proline uptake and after 10 min of proline uptake.

Sample	ATP		
	Initial	After 5 min	After 10 min
Control	9.55 $\pm$ 0.18	15.64 $\pm$ 0.47	16.73 $\pm$ 0.05
Iodoacetate	8.37 $\pm$ 0.13	12.99 $\pm$ 0.17	10.42 $\pm$ 0.30
KCN	8.69 $\pm$ 0.16	7.05 $\pm$ 0.06	5.92 $\pm$ 0.05

The respiratory inhibitor KCN (1 mM) had less effect on proline uptake (Table 2), but it induced a steady decrease in intracellular ATP concentration (Table 3) when the cells were transporting proline. Consequently, both inhibitors interfere with proline transport and ATP production, but in different ways.

In order to determine whether iodoacetate reacted directly with the proline transport protein, protection of the transporter by proline was tested. A high concentration of the substrate (5 mM) was added to the cell suspension before incubation with 2 mM iodoacetate, in parallel with a control batch (no inhibitor) and a batch with iodoacetate but no proline. After 7 min at room temperature, the cell suspensions were washed twice in PSGK buffer and proline uptake experiments were performed as described above. No significant protection ($n = 3$, $P < 0.01$) of the transport protein by its substrate was observed ($54.9 \pm 7.9\%$ of control uptake without protection and $68.4 \pm 3.1\%$ with 5 mM proline). Consequently, iodoacetate seems to react either outside the substrate-binding site of the transporter or with other proteins connected to the proline transporter. Another thiol reagent, iodoacetamide, tested in similar experimental conditions, did not induce any decrease in proline transport ($105 \pm 2.2\%$ of control activity).

Two inhibitors of glutamate semialdehyde dehydrogenase, the enzyme catalysing the second step of proline breakdown, were tested at a concentration of 2 mM in the cell suspension. The inhibitors, valerolactam and 5-amino-n-valeric acid, had no effect on proline transport even after a long incubation period (10 min).

Proline uptake experiments were performed on *T. brucei* membrane vesicles in order to verify the ATP-dependence of the transport system. Preliminary results showed a proline uptake of 0.76 ± 0.02 nmol/mg of protein ($n = 5$) after a 20 s incubation in the presence of 5 mM ATP inside and outside the vesicles. Extravesicular ATP (2 mM) did not induce any proline uptake (no net uptake detected; S.D. 0.08 nmol/mg, $n = 3$). The same result was obtained without ATP (0.003 ± 0.07 nmol/mg; $n = 3$).

DISCUSSION

The results obtained for L-proline uptake in the procyclic form of *T. brucei* showed the active nature of this process, as proved by the high concentration gradient after 1 min of uptake and the sensitivity to metabolic inhibitors, sodium acetate and KCN.

The concentration-dependent transport kinetics exhibit Michaelis-Menten kinetics, thus typical for a single carrier-mediated transport system (linearization upon double-reciprocal transformation). The K_m value of 19 μ M for proline uptake in *T. brucei* is lower than that found in *Leishmania* promastigotes (Bonay and Cohen, 1983), 80 μ M. The V_{max} value of

17 nmol/min per 10^8 cells obtained for *T. brucei* is higher than that found in *Leishmania*. Digitonin-permeabilized cells were still found to accumulate some ^{14}C label. Since it was shown that only an extremely low amount of proline is incorporated into proteins, a possible explanation is that proline or its metabolites may be concentrated in organelles, which are not permeabilized at the digitonin concentration used.

The activation energy, E_a , of $74.9 \text{ kJ} \cdot \text{mol}^{-1}$ was found to be slightly lower than that of $95 \text{ kJ} \cdot \text{mol}^{-1}$ observed in human Caco-2 cells for proline uptake (Nicklin et al., 1992), but it is well within the usual range for carrier-mediated processes, $29\text{--}105 \text{ kJ} \cdot \text{mol}^{-1}$ (Hoefer, 1977). The bloodstream form of *T. brucei* also concentrated proline, but to a lesser extent than the procyclic form. These results are in agreement with proline being the main carbon source of the procyclic form. The utilization of proline by the bloodstream form seems to be less essential.

Experiments showed that proline uptake is not energized via co-transport with Na^+ , K^+ or H^+ .

The transport of proline and the concentration of cellular ATP were both affected by an admittedly high concentration of iodoacetate (10 mM) and by 1 mM KCN, but more than 50 % of transport activity was maintained after 10 min of incubation with either inhibitor. The results indicate that incubation by iodoacetate affected first proline uptake and, only later, ATP concentration. In contrast, inhibition of respiration by KCN seemed to decrease rapidly ATP concentration and, as a consequence, proline uptake, suggesting the energy dependence of the transport system. Moreover, KCN has been shown to inhibit the first step of proline breakdown, the oxidation of proline (Strecker, 1971). Consequently, even if proline uptake activity is only partially affected by KCN, transported proline cannot be utilized by the cell to produce ATP.

In order to demonstrate that proline transport is ATP-dependent in *T. brucei*, uptake experiments on plasma-membrane vesicles of the cells were performed. The results thus obtained showed that proline is accumulated in the vesicles when ATP is present simultaneously inside and outside the vesicles. No transport was observed when ATP was not added to the suspension or when it was only extravesicular. This experiment confirms that intracellular ATP is necessary for the uptake of proline by *T. brucei* cells.

In *Leishmania* promastigotes, despite a different transport system (protonmotive-force-driven), proline uptake is also inhibited by azetidine-2-carboxylate and alanine (Bonay and Cohen, 1983), showing a similarity of substrate specificity for the carriers of both species. Alanine is the main product of proline breakdown, via glutamate oxidation and pyruvate transamination in *Leishmania* (Bonay and Cohen, 1983) and presumably also in the procyclic form of *T. brucei* (Evans and Brown, 1972). Moreover, the *T. brucei* procyclic stage in the stationary phase of growth has been shown to utilize glucose (although proline is its preferred carbon source) and to release alanine, by contrast with the bloodstream form, which releases

only pyruvate (Evans and Brown, 1972). The present study showed that glutamate is the only detectable radiolabelled product of proline breakdown in the cell extracts. This result shows that the final product, alanine, is probably released to regulate the cell volume, as in other trypanosomatids (Blum, 1992).

The Na^+ -independent transport system characterized in the present study appears to be very specific, since only two amino acids, alanine and cysteine, are competitive inhibitors. Competition studies on other amino acid carriers revealed transport systems having less structural specificity, for instance in *T. gambiense* (Southwork and Read, 1972; Hansen, 1979), in *T. equiperdum* (Ruff and Read, 1974) and in *T. cruzi* (Hampton, 1970; Goldberg et al., 1976). Thus the high specificity of the proline carrier system shows the essential role of proline in the metabolism of *T. brucei*. Furthermore, Na^+ -independent carriers are usually not highly concentrative, by contrast with Na^+ -dependent systems (Christensen, 1984). Among Na^+ -independent carriers, the proline carrier in *T. brucei* does not resemble the systems L and T usually found in vertebrate cells. Consequently, the proline carrier characterized in *T. brucei* seems to represent a new type of Na^+ -independent amino acid transport system.

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REFERENCES

- Baltz, T., Baltz, D., Giroud, Ch. and Crockett, J. (1985) *EMBO J.* **4**, 1273–1277
- Blum, J. J. (1992) *J. Cell. Physiol.* **152**, 111–117
- Bonay, P. and Cohen, B. E. (1983) *Biochim. Biophys. Acta* **731**, 222–228
- Brun, R. and Schonenberger, M. (1979) *Acta Trop.* **36**, 289–292
- Christensen, H. N. (1984) *Biochim. Biophys. Acta* **779**, 255–269
- Evans, D. E. and Brown, R. C. (1972) *J. Protozool.* **19**, 686–690
- Glaser, T. A. and Mikkada, A. J. (1992) *Mol. Biochem. Parasitol.* **51**, 1–8
- Goldberg, S. S., Silva Pereira, A. A., Chiari, E., Mares-Guia, M. and Gazzinelli, G. (1976) *J. Protozool.* **23**, 179–186
- Hampton, J. R. (1970) *Int. J. Biochem.* **1**, 706–714
- Hansen, B. D. (1979) *Exp. Parasitol.* **48**, 296–304
- Hoefer, M. (1977) *Transport durch Biologische Membranen*, Verlag Chemie, Weinheim
- Johnson, A. B. and Strecker, H. J. (1962) *J. Biol. Chem.* **237**, 1876–1882
- Kiari, J. K. and Njogu, R. M. (1983) *Int. J. Biochem.* **15**, 1379–1383
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nakanishi, T., Turner, R. J. and Burg, M. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6002–6006
- Nicklin, P. L., Irwin, W. J., Hassan, I. F. and Mackay, M. (1992) *Biochim. Biophys. Acta* **1104**, 283–292
- Rovis, L. and Baekkeskov, S. (1980) *J. Parasitol.* **80**, 507–524
- Ruff, M. D. and Read, C. P. (1974) *J. Protozool.* **21**, 368–373
- Southwork, G. C. and Read, C. P. (1972) *Comp. Biochem. Physiol.* **41A**, 905–911
- Strecker, H. J. (1971) *Methods Enzymol.* **17B**, 251–254
- Zilberstein, D. and Dwyer, D. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1716–1720
- Zingales, B., Carniol, C., Abraham, P. A. and Colli, W. (1979) *Biochim. Biophys. Acta* **550**, 233–244