The inhibition of pyruvate transport across the plasma membrane of the bloodstream form of *Trypanosoma brucei* and its metabolic implications

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The pyruvate produced by glycolysis in the bloodstream form of the trypanosome is excreted into the host bloodstream by a facilitated diffusion carrier. The sensitivity of pyruvate transport for α-cyano-4-hydroxycinnamate and the compound UK5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate], which are known to be selective inhibitors of pyruvate (monocarboxylate) transporters present in mitochondria and the plasma membrane of eukaryotic cells, was examined. The trypanosomal pyruvate carrier was found to be rather insensitive to inhibition by α-cyano-4-hydroxycinnamate (K_i = 17 mM) but could be completely blocked by UK5099 (K_i = 49 μM). Inhibition of pyruvate transport resulted in the retention, and concomitant accumulation, of pyruvate within the trypanosomes, causing acidification of the cytosol and osmotic destabilization of the cells. Our results indicate that this physiological state has serious metabolic consequences and ultimately leads to cell death; thereby identifying the pyruvate carrier as a possible target for chemotherapeutic intervention.

INTRODUCTION

During its parasitic life cycle the protozoan *Trypanosoma brucei* passes through at least two distinct morphological and physiological stages, i.e. the procyclic (insect) form in the digestive tract of the tsetse fly and the bloodstream-dwelling trypomastigote form in vertebrates. The bloodstream form of the trypanosome relies for its energy supply exclusively on glycolysis which takes place in the glycosome, an organelle characteristic for the order of the Kinetoplastida. In *T. brucei* the organellar sequestering of glycolytic enzymes enables an unsurpassed glycolytic rate of 85 nmol of glucose·min^{-1}·(mg of protein)$^{-1}$ at 37 °C [1,2]. Under normal aerobic conditions glucose, taken up from the blood, is converted into pyruvate which, due to the absence of a functional Krebs cycle and the lack of lactate dehydrogenase, is not metabolized any further and is thus excreted into the host's bloodstream [3,4]. Simple diffusion seems insufficient to be responsible for the pyruvate efflux because pyruvate (pK_a = 2.49) at physiological pH is mainly present in its dissociated form, which is assumed to be unable to freely permeate through biological membranes. Recently kinetic studies showed that the extrusion of pyruvate across the plasma membrane is mediated by a specific facilitated diffusion carrier [5,6]. Not much is known yet about the molecular mechanism of this transport process; however, to prevent acidification of the cytosol the transport of the pyruvate anion must be coupled to the co-transport of a proton, as is described for other pyruvate carriers (see reviews [7–9]). It was hypothesized that the efflux of more than one proton could chemiosmotically couple the obligatory pyruvate efflux to the generation of metabolic energy, cf. the situation found in some bacterial species [10]. However, no electrochemical proton gradient over the plasma membrane could be detected in bloodstream-form trypanosomes [11], implying that the energy potential contained in the transmembrane pyruvate gradient is not used by the trypanosome. With regard to the substrate specificity a clear distinction could be made between the trypanosomal pyruvate carrier and other pyruvate (monocarboxylate) carriers located in the mitochondrial inner membrane and the plasma membrane of other eukaryotic cells [7–9,12], in that it does not transport L-lactate [5].

The α-cyanocinnamic acid-derived inhibitors have been instrumental in detailed kinetic analyses and biochemical characterization of various monocarboxylate transporters [7–9], in particular the mitochondrial pyruvate transporter [13–15], by elucidating its molecular mechanism [15,16] and the isolation of the carrier (see, for example, [17,18]). To gain a better understanding of the biochemical characteristics and physiological significance of the trypanosomal pyruvate carrier we examined its sensitivity for these inhibitors.

MATERIALS AND METHODS

Growth and isolation of trypanosomes

Bloodstream forms of *Trypanosoma brucei* stock 427 were grown in 300 g Wistar rats. Blood was diluted with PBS, pH 8.0, containing 1 % (w/v) glucose (PSG). A buffy coat was obtained by centrifugation at 300 *g* for 15 min, the band of concentrated trypanosomes was removed, pelleted and resuspended in PSG. Remaining blood constituents were removed by filtration through a small DEAE-cellulose column essentially according to the methods of Lanham and Godfrey [19] and Opperdoes et al. [20]. Subsequently the cells were washed once with PSG and kept as a stock suspension in PSG on ice [cell density of (3–6) × 10^8 trypanosomes·ml^{-1}]. Routinely before and during the experiment trypanosomes were examined by phase-contrast microscopy and the number of live cells determined by counting in a Petroff–Hausser cell.

Influx experiments

Pyruvate uptake experiments were performed, using the silicone-oil centrifugation technique, essentially as described previously [5,21]. Briefly, trypanosomes were transferred to the incubation medium by taking an aliquot (1100 μl) from the stock suspension, which was washed once and finally resuspended in an equal volume of glucose serum lactalbumin haemoglobin (GSLH)

Abbreviations used: HBSS, Hanks balanced salt solution; GSLH, glucose serum lactalbumin haemoglobin; PSG, PBS (pH 8.0) containing 1 % (w/v) glucose.

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medium [22], prewarmed to 37 °C. This medium was supplemented with extra glucose till a final concentration of 10 mM was reached (GSLH/glucose). The concentration of pyruvate was varied and substances of which the effect upon pyruvate uptake had to be tested were added as indicated in the various experiments. Alternatively an albumin-free medium was used consisting of Hanks balanced salt solution (HBSS: 1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$ and 0.3 mM Na_2HPO_4) supplemented with 15 mM Hepes, pH 7.4, and 10 mM glucose (HBSS/Hepes/glucose). The trypanosomes were allowed to preincubate in the medium for 30 s at 37 °C. The cell density in each experiment was $(3–6) \times 10^8$ trypanosomes $\cdot ml^{-1}$. The uptake was initiated by the addition and instant mixing of a 2.5–5 μl spike of [^{14}C]pyruvate (0.05 $\mu Ci \cdot \mu l^{-1}$) and [^{3}H]inulin (0.05 $\mu Ci \cdot \mu l^{-1}$). The reaction was stopped after 45 s (the longest time over which pyruvate uptake is linear with time [5]) by rapidly centrifuging the cells, in 400 μl microfuge tubes, through 75 μl of a 3:1 mixture of 1-bromododecane and Serva DC200 silicone fluid. Usually each data point consisted of four replicate tubes, each containing 250 μl of incubation medium. The radioactivity associated with the cell pellet or retained in the supernatant was determined by $^{14}C/^3H$ dual label counting; the [^{3}H]inulin was used to correct for the extracellular space as described by Ter Kuile and Opperdoes [21].

Efflux experiments

After an initial wash with GSLH/glucose the trypanosomes [cell density $(3–6) \times 10^8$ trypanosomes $\cdot ml^{-1}$] were resuspended in GSLH/glucose containing 2.5 mM pyruvate, [^{14}C]pyruvate (0.25 $\mu Ci \cdot ml^{-1}$) and [^{3}H]inulin (0.25 $\mu Ci \cdot ml^{-1}$). The radiolabelled pyruvate was allowed to equilibrate with the cells for 1.5 min at 37 °C after which the cells were washed once with ice-cold GSLH/glucose medium and resuspended in GSLH/glucose containing either no or variable amounts of UK5099 (100–1000 μM). The efflux of pyruvate at 37 °C was monitored at 20–30 s intervals by the silicone-oil centrifugation technique [5,21], with the exception that aliquots of 200 μl were directly layered on top of the mixture of organic fluids as described above.

ΔpH, intra- and extracellular volume measurements

Trypanosomes were treated exactly as described for influx experiments except that after the 30 s preincubation period 10 μl of [^{14}C]methylamine (0.1 $\mu Ci \cdot \mu l^{-1}$), ^3H_2O (1 $\mu Ci \cdot \mu l^{-1}$) or [^{3}H]inulin (0.05 $\mu Ci \cdot \mu l^{-1}$) were added and directly mixed with the incubation medium. Equilibration of the radioactive probes between the external medium and the cell interior was allowed to proceed for 1.5 min at 37 °C.

The internal volume was determined with ^3H_2O, using [^{3}H]inulin, in an identical incubation, to correct for the extracellular water volume. The internal volume of *T. brucei* cells was approx. 2 $\mu l/10^8$ cells.

The internal pH was determined from the distribution of [^{14}C]methylamine [23] and the ΔpH calculated using the following formula:

$$pH_{in} = pH_{out} - \log([\text{methylamine}]_{in}/[\text{methylamine}]_{out})$$

It was previously demonstrated that [^{14}C]methylamine reaches its equilibrium distribution in 1–1.5 min under these conditions after which there is no additional uptake of the probe, indicating that methylamine is not metabolized by the trypanosomes [11].

Miscellaneous

Pyruvate concentrations were routinely measured enzymically in a mixture containing 50 mM potassium phosphate buffer, pH 7.4, 0.1% (v/v) Triton X-100, 0.4 mM NADH and 2.5 units of lactate dehydrogenase (Boehringer, Mannheim, Germany) at 25 °C.

The rate of O_2 consumption at 37 °C was measured polarographically with a Clark-type oxygen electrode. In a typical experiment a suspension in GSLH/glucose of 0.5×10^7 bloodstream-form trypanosomes $\cdot ml^{-1}$ was used.

Stock solutions of the inhibitors were freshly prepared either in *N,N*-dimethylformamide (for UK5099) or in the incubation medium itself. The pH of the α-cyano-4-hydroxycinnamic acid stock solution was set to neutral pH by titration with NaOH. Stock solutions were diluted such that the concentration of solvent was similar in all incubations and never exceeded 1% (v/v). A control incubation containing 1% (v/v) of *N,N*-dimethylformamide was routinely included and was found to have no measurable effect on pyruvate transport.

Chemicals

The compound UK5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate] was a generous gift from Pfizer Ltd., Sandwich, Kent, U.K. [2-^{14}C]Pyruvic acid (approx. 30 mCi $\cdot$ mmol^{-1}), [^{3}H]inulin (355 mCi $\cdot g^{-1}$), ^3H_2O (1 mCi $\cdot g^{-1}$) and [^{14}C]methylamine (50 mCi $\cdot$ mmol^{-1}) were purchased from Du Pont de Nemours (New England Nuclear Division, Dreieich, Germany). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were of the purest grade available.

RESULTS

Effect of α-cyano-4-hydroxycinnamate and the compound UK5099 on trypanosomal pyruvate transport

Pyruvate (monocarboxylate) transport across the plasma mem-

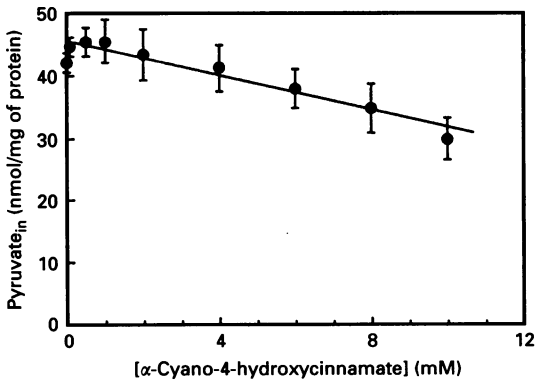

Figure 1 The effect of α-cyano-4-hydroxycinnamate on the uptake of pyruvate by *T. brucei*

The uptake of pyruvate after 45 s was determined in albumin-free HBSS supplemented with 15 mM Hepes, pH 7.2, and 10 mM glucose (HBSS/Hepes/glucose) and containing 2.5 mM pyruvate and increasing amounts of α-cyano-4-hydroxycinnamate. Depicted are the average values of two independent experiments involving different trypanosome preparations, error bars indicate 2 × S.D. ($n = 6–8$). An apparent K_i value of 17 mM was calculated, using linear regression, from the data presented in this Figure, after plotting $1/v$ against the inhibitor concentration (Dixon plot). The actual extent of carrier-dependent uptake rates was corrected for the presence of a diffusion component which was estimated to be 17.5% of the control uptake rate under these conditions.

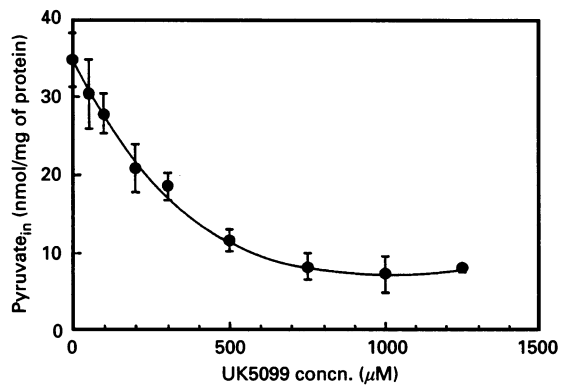

Figure 2 The compound UK5099 inhibits pyruvate influx by the pyruvate carrier in the plasma membrane of *T. brucei*

The uptake of pyruvate was determined after 45 s in GSLH/glucose containing 2 mM pyruvate and various concentrations of the compound UK5099. Depicted are the average values of 3–4 independent experiments involving different trypanosome preparations, error bars indicate 2 × S.D. (n = 7–16). An apparent K_i value of 49 μM was calculated, using linear regression, from the data presented in this Figure, after plotting $1/v$ against the inhibitor concentration (Dixon plot). The actual extent of carrier-dependent uptake rates was corrected for the presence of a diffusion component which was estimated to be 17.5% of the control uptake rate under these conditions.

Table 2 Increased cytosolic pyruvate concentration and acidification of the cell interior in *T. brucei* bloodstream-form trypanosomes treated with the compound UK5099

Trypanosomes were incubated for 120 s at 37 °C in GSLH/glucose in the absence or presence of increasing concentrations of UK5099 (50–1250 μM). The decrease in transmembrane ΔpH ($pH_{in} - pH_{out}$), due to the retention of pyruvate in the intracellular compartment as a function of the UK5099 concentration, was measured by the equilibrium distribution of the weak base [^{14}C]methylamine as described in the Materials and methods section. Presented are the average values ± S.D. of two independent experiments (n = 8) involving different trypanosome isolations. For the determination of the cytosolic pyruvate concentration the cells were rapidly centrifuged through a layer of silicon oil, cooled in ice-water and frozen in liquid nitrogen. Cell pellets were cut from the tubes with a razor blade and dissolved in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, after which the pyruvate concentration was determined. The cytosolic pyruvate concentration was calculated by taking into account the intracellular volume, which was measured simultaneously, and by assuming the cytosol occupies a volume percentage of 48.8% according to Coppens et al. [25]. The results depicted are the average values ± S.D. of two independent experiments (n = 4), involving different preparations of trypanosomes.

UK5099 (μM)	ΔpH	Cytosolic pyruvate concn. (mM)
0	0.180 ± 0.023	26.37 ± 4.48
50	−0.269 ± 0.051	19.30 ± 0.56
100	−0.259 ± 0.041	28.04 ± 3.77
200	−0.351 ± 0.054	36.38 ± 4.47
300	−0.463 ± 0.027	46.16 ± 3.15
500	−0.494 ± 0.020	57.82 ± 4.72
750	−0.469 ± 0.131	22.22 ± 5.58
1000	−0.285 ± 0.141	ND
1250	−0.152 ± 0.027	ND

brane in different eukaryotic cell types and across the mitochondrial inner membrane is known to be inhibited by a group of potent and relatively specific inhibitors: the α-cyanocinnamic acid derivatives ([13,14], see also reviews [7–9]). The compounds presumably act as pyruvate analogues by binding to the active site of the transporter [16]. Two representatives of this class of inhibitors, i.e. α-cyano-4-hydroxycinnamate and the compound UK5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate], were tested for their ability to interfere with trypanosomal pyruvate transport. Figure 1 depicts the rate of pyruvate uptake as a function of the α-cyano-4-hydroxycinnamate concentration. An increasing inhibitor concentration gives rise to a decrease in pyruvate uptake. However, the affinity of the inhibitor for the pyruvate carrier seems rather low (cf. Figure 2) as relatively high concentrations (apparent K_i = 17 mM) are needed to obtain a significant inhibitory effect. These results are in agreement with data from Barnard et al. [6] who reported an inhibitory effect of α-cyano-3-hydroxycinnamic acid on the pyruvate efflux from trypanosomes. The experiments were performed in an albumin-free medium (HBSS/Hepes/glucose), as it is reported in the literature that the inhibitor complexes with albumin and/or other serum proteins and loses its inhibitory capability [24]. In an identical experiment, using the albumin-containing medium GSLH/glucose, we found that the inhibitory effect of α-cyano-4-hydroxycinnamate was slightly smaller; the apparent K_i value being only two times greater (results not shown).

A more effective inhibitor, as judged from Figure 2, is UK5099, a close analogue of α-cyanocinnamate but with a larger aromatic group (see [14] for its structure formula). Addition of this inhibitor to our influx assay showed that it is able to almost completely block the uptake of pyruvate when present at a 1 mM concentration (Figure 2) (apparent K_i = 49 μM). The small residual uptake of approx. 20% of the control uptake value, presumably represents uptake by diffusion as has been demonstrated to occur [5]. The experiments of Figure 2 were performed in GSLH/glucose. The use of HBSS/Hepes/glucose instead, did not significantly alter the potency of the inhibitor (results not shown).

In addition the effect of UK5099 on pyruvate efflux was

Table 1 The compound UK5099 inhibits pyruvate efflux from bloodstream-form *T. brucei*

The efflux of [^{14}C]pyruvate from preloaded cells was determined as a function of time when cells were resuspended in GSLH/glucose in the absence or presence of 200 μM, 500 μM and 1 mM UK5099. Shown are the average percentages ± S.D. (n = 3) of [^{14}C]pyruvate counts found retained in the cells in three independent experiments involving different trypanosome preparations.

	Pyruvate retained (%)			
Efflux time (s)	No UK5099	200 μM UK5099	500 μM UK5099	1 mM UK5099
20	49.92 ± 2.22	63.23 ± 1.39	78.82 ± 4.13	92.25 ± 1.18
50	34.96 ± 0.42	44.15 ± 5.00	61.58 ± 8.55	71.30 ± 8.91
80	27.29 ± 2.78	30.67 ± 4.99	47.82 ± 6.87	47.26 ± 18.75
110	20.30 ± 1.51	27.02 ± 5.59	40.61 ± 10.48	18.66 ± 16.32
140	18.28 ± 1.36	21.94 ± 4.87	33.33 ± 10.07	14.49 ± 13.91

Table 3 Respiration rate of bloodstream-form *T. brucei* decreases with increasing UK5099 concentrations

The oxygen consumption was determined of freshly isolated bloodstream-form trypanosomes suspended in GSLH/glucose in the absence or presence of various concentrations of the inhibitor UK5099. The results depicted are the average values ± S.D. measured in two independent experiments ($n = 4$), involving different trypanosome preparations. The respiration rate in the absence of UK5099 is arbitrarily set at 100% and corresponds to 67.55 ± 16.11 μmol of $O_2 \cdot min^{-1} \cdot mg$ of $protein^{-1}$ ($n = 20$).

UK5099 (μM)	Respiration rate (% of control)
0	100
200	58.87 ± 5.4
500	27.15 ± 5.6
700	0.0
1000	0.0

determined. As is shown in Table 1 the rate and extent of the pyruvate efflux from preloaded trypanosomes was dependent on the UK5099 concentration present in the medium. The higher the UK5099 concentration the lower the overall efflux rate. When the UK5099 concentration reached 1 mM the cells apparently became leaky after approx. 50 s and released their contents into the surrounding medium. The cells, however, did not completely loose their integrity as they still could be pelleted through the silicon-oil layer, but a prolonged centrifugation time was necessary. The exact moment the cells fail to retain pyruvate varied between different trypanosome preparations. This is clearly reflected by the large error values in the 1 mM UK5099 incubation of Table 1.

The inhibition of the pyruvate carrier by UK5099, preventing the influx as well as the efflux of pyruvate, has been clearly demonstrated in Figure 2 and Table 1, and must necessarily lead to the retention and accumulation of pyruvate within the cells provided that glycolysis is not inhibited.

Inhibition of the pyruvate transport by UK5099 acidifies the cell interior

Accumulation of pyruvate acidifies the cytosol as is demonstrated in Table 2. By determining the equilibrium distribution of the weak base [^{14}C]methylamine between the external medium and the cell interior the transmembrane ΔpH could be calculated. When pyruvate efflux was impaired by UK5099 the internal pH dropped from 7.2, when no inhibitor was present, to approx. 6.8. Already, at an inhibitor concentration of 750 μM, the trypanosomes became affected so badly that the pH probe could not be retained and was lost from the cells, explaining the decrease in ΔpH at elevated inhibitor concentrations. Note that, in this particular experimental set-up, the cells were incubated for a total of 120 s with the inhibitor, in contrast to only 75 s in the experiments presented in Figure 2.

We also attempted to measure the actual cytosolic concentrations of pyruvate in trypanosomes that are actively metabolizing glucose and of which the extrusion of pyruvate, by the pyruvate carrier, is inhibited by UK5099. From the results presented in Table 2 it can be inferred that the internal pyruvate concentration can reach very high levels. An internal concentration of almost 60 mM pyruvate was measured in trypanosomes incubated for 120 s in the presence of 500 μM UK5099. The observed decrease in pyruvate concentration at 750 μM UK5099 may again be explained by assuming that the cells become leaky (cf. Tables 1 and 2). Pyruvate could not be assayed in cells treated with the highest inhibitor concentration because the UK5099 remained associated with the pelleted 'leaky' cells and inhibited the activity of lactate dehydrogenase required for the assay (E. A. C. Wiemer, P. A. M. Michels and F. R. Opperdoes, unpublished work).

The experiments in Figure 2 and Tables 1 and 2 show a good correlation between the inhibition of pyruvate transport in both directions across the trypanosome plasma membrane, the increase of internal pyruvate concentration and the decrease of intracellular pH. Both the high internal pyruvate levels and

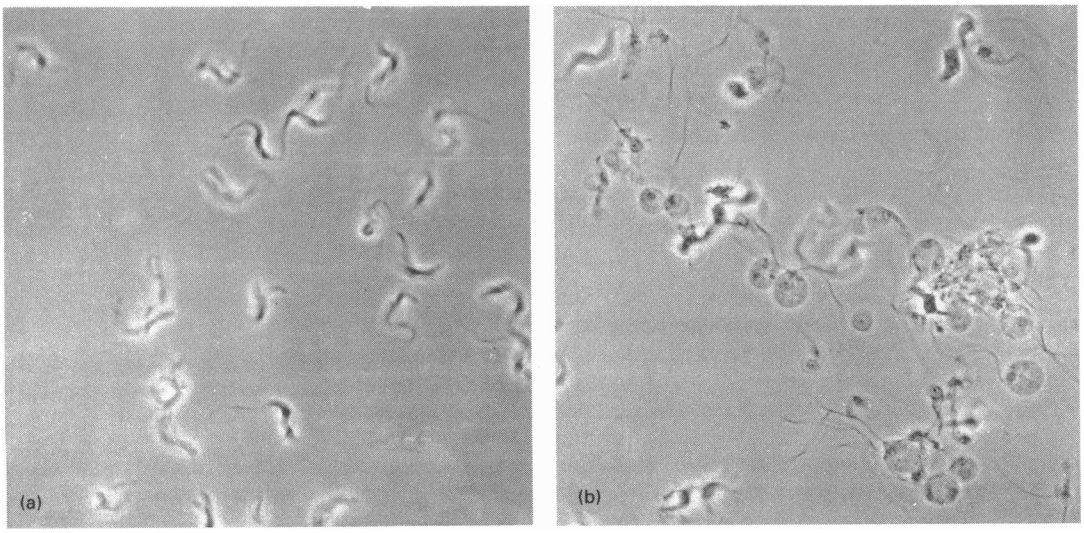

Figure 3 Morphological evidence that inhibition of the pyruvate carrier by UK5099 in the bloodstream form of *T. brucei* leads to cell lysis

Freshly isolated bloodstream-form trypanosomes were resuspended in GSLH/glucose at a concentration of 3.5×10^8 cells $\cdot ml^{-1}$ containing (**a**) no UK5099, or (**b**) 500 μM UK5099 and were kept at 37 °C. At regular intervals the motility and morphological features of the trypanosomes were examined by phase-contrast microscopy. Shown are photomicrographs of the cellular appearance after an incubation of 12 min.

consequent cytosolic acidification must have severe metabolic consequences for the cell.

Biochemical and morphological assessment of the physiological consequences of inhibition of the pyruvate carrier by UK5099

The long-term effects of a severely impaired pyruvate carrier were assessed by following the rate of oxygen consumption after a 30 s preincubation of the cells with the inhibitor UK5099. The data, summarized in Table 3, show that respiration is inhibited by 41.13% if 200 μM UK5099 is present. Higher concentrations cause a further decline of the respiration rate which stabilizes at approx. 27.15% of the control rate at 500 μM UK5099. No oxygen consumption could be measured, in cell suspensions treated with 700–1000 μM of the inhibitor.

Additional evidence that blockage or inhibition of the pyruvate carrier by UK5099 eventually causes cell death is presented in Figure 3. Trypanosomes were incubated for prolonged times (up to 20 min) in GSLH/glucose containing different concentrations of the inhibitor UK5099. Figure 3(a) shows a control incubation in which the trypanosomes were incubated for 12 min in GSLH/glucose; the cells were motile and did not appear to be swollen or lysed. Upon incubation with UK5099 (see Figure 3b) most of the cells appeared to swell and often obtained a balloon-like appearance; initially these cells were still motile, but lost their motility after a while and finally lysed forming clumps of dead cells. In order to exclude that UK5099 has an aspecific effect on membranes causing them to rupture, we incubated procyclic trypanosomes with the inhibitor and observed that they did not swell or lyse (results not shown).

DISCUSSION

In the bloodstream-form trypanosome the pyruvate produced by glycolysis is excreted into the host's bloodstream, a process that involves the action of a facilitated diffusion carrier [5,6]. The effects of inhibitors of pyruvate (monocarboxylic) transporters on trypanosomal pyruvate transport were determined. Our results indicated that the pyruvate carrier is of vital importance for the bloodstream-form trypanosome.

The α-cyanocinnamate derivatives α-cyano-4-hydroxycinnamate and UK5099 were shown to inhibit trypanosomal pyruvate transport. Particularly, UK5099 proved to be rather effective. Addition of UK5099 to bloodstream-form trypanosomes impaired or totally prevented carrier-mediated pyruvate transport across the plasma membrane. Glucose uptake and glycolysis are not directly affected by UK5099, as pyruvate continues to be produced; a fact emphasized by its accumulation within the cells. This observation is of crucial importance because it argues against the possibility that, under the experimental conditions used, UK5099 reacts with glycolytic proteins in addition to the pyruvate transporter. A scenario like that has recently been reported to occur by Barnard et al. [6], who showed that the alkylating agent, and competitive inhibitor of trypanosomal pyruvate transport, bromopyruvate bound to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, present in the glycosome, thereby inhibiting the glycolytic flux. The result being a severely reduced production of pyruvate which subsequently leads to a slowing down of pyruvate efflux. It was observed that pyruvate efflux eventually stopped at bromopyruvate concentrations that only inhibited pyruvate uptake by approx. 50%.

The experiments involving UK5099 were performed in the albumin-containing medium GSLH/glucose. This medium resembles the physiological environment of the trypanosome more closely than HBSS/Hepes/glucose. This may be especially important since partial inhibition or blockage of the pyruvate export is clearly detrimental for the cells. In the literature it is described [24] that albumin, and other serum proteins, may bind the hydrophobic α-cyanocinnamate derivatives and hence lower the free inhibitor concentration that is able to interact with the pyruvate transporter. However, under our experimental conditions the use of an albumin-free medium did not significantly alter the potency of the inhibitor (results not shown).

It was also noted that the inhibitory effect of UK5099 tended to be enhanced at lower pH (< 7.4), i.e. lower inhibitor concentrations were needed to obtain the maximal inhibitory effect. It is not yet clear whether this phenomenon has a biological origin or is due to an intrinsic property of the inhibitor itself.

The metabolic consequences for the trypanosome, when pyruvate export is partially or totally blocked, are considerable. Cytosolic pyruvate levels were measured to rise at least as high as 57 mM in cells treated with 500 μM UK5099 compared with approx. 26.3 mM under normal circumstances. Furthermore a definite acidification of the cytosol could be demonstrated as a result of the entrapment of pyruvate. The acidification, about 0.5 pH unit, as well as the increased internal pyruvate levels may slow down or block glycolysis, depriving the cell of its only source of energy. Alternatively the high pyruvate concentration may osmotically destabilize the cells, causing them to counteract the osmotic gradient by taking up water. Such an compensatory action would explain the clearly swollen and lysed cells that were observed when cells were treated with UK5099. The apparent permeability of the plasma membrane for pyruvate (Tables 1 and 2) is most likely due to osmotic stress. The reduced retention of methylamine at high UK5099 concentrations may be the combined result of a leaky plasma membrane and the decrease in acidity of the cell interior. The leakiness of the plasma membrane was verified by a simple Trypan Blue exclusion experiment in which it was shown that the UK5099-treated cells, in contrast to untreated cells, were not able to exclude the dye from entering the cytoplasm (results not shown). Swelling and lysis did not occur when the procyclic (insect) form of *T. brucei* was treated with the inhibitor, reflecting its different metabolic make-up. In the procyclic life-cycle stage the pyruvate produced in glycolysis is not excreted, but is instead shuttled into the mitochondrion and further oxidized.

Whatever the precise explanation of the observed effects, it is clear that the cells quite rapidly cease to respire and presumably die. In our opinion it is imperative from the results described in this paper that the pyruvate export is carrier-mediated and that this carrier is of vital importance for the cell. In addition, the fact that the pyruvate transporter is located in the plasma membrane and thus readily accessible for externally added substances clearly marks it as a likely target for drugs against trypanosomiasis. The α-cyanocinnamic acid derivatives might not be very useful in this respect as they seem to interact with most mammalian monocarboxylate transporters. Further studies should reveal as to how far the trypanosomal carrier and the host monocarboxylate carrier have diverged.

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