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Purine nucleobase transport in bloodstream forms of *Trypanosoma brucei brucei* is mediated by two novel transporters

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Abstract

The mechanism and inhibitor sensitivity of hypoxanthine transport by bloodstream forms of *Trypanosoma brucei brucei* was investigated. The dose response curve for the inhibition of hypoxanthine transport (1 μM) by guanosine was biphasic; $\approx 90\%$ of transport activity was inhibited with a K_i value of 10.8 ± 1.8 μM, but 10% of the activity remained insensitive to concentrations as high as 2 mM. These two components of hypoxanthine transport are defined as guanosine-sensitive (H2) and guanosine-insensitive (H3). Hypoxanthine influx by both components was saturable, but there was a marked difference in their K_m values (123 ± 15 nM and 4.7 ± 0.9 μM for H2 and H3, respectively) although the V_{max} values (1.1 ± 0.2 and 1.1 ± 0.1 pmol $(10^7$ cells$)^{-1}$ s^{-1}, $n = 3$) were similar. Hypoxanthine uptake via the H2 carrier was inhibited by purine bases and analogues as well as by some pyrimidine bases and one nucleoside (guanosine), whereas the H3 transporter was sensitive only to inhibition by purine nucleobases. H2-mediated hypoxanthine uptake was inhibited by ionophores, ion exchangers and the potential H^+-ATPase inhibitors, *N*,*N*′-dicyclohexylcarbodiimide (DCCD) and *N*-ethylmaleimide (NEM). Measurements of the intracellular pH and membrane potential of bloodstream trypanosomes in the presence and absence of these agents established a linear correlation between protonmotive force and rate of [^{3}H]hypoxanthine (30 nM) uptake. We conclude that hypoxanthine transport in bloodstream forms of *T. b. brucei* occurs by two transport systems with different affinities and substrate specificities, one of which, H2, appears to function as a H^+/hypoxanthine symporter. © 1997 Elsevier Science B.V.

Keywords: Trypanosome; Purine transporter; Nucleobase uptake; Protonmotive force; Hypoxanthine

Abbreviations: BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester; Bisoxonol, bis-(1,3-diethylthiobarbituric acid)trimethine oxonol; CCCP, carbonyl cyanide chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 3-(*N*-morpholino)propane-sulfonic acid; NEM, *N*-ethylmaleimide; pH_i, intracellular pH; pH_o, extracellular pH; V_m, membrane potential.

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1. Introduction

Protozoan parasites belonging to the *Trypanosoma brucei* subgroup are the causative agents of sleeping sickness in man and nagana in cattle. In mammals, *T. b. brucei* exist in the bloodstream, whereas in the Tsetse fly they exist in the midgut, as procyclic forms. Many of the existing drugs to treat African sleeping sickness cause severe side-effects and their effectiveness is undermined by the emergence of drug-resistant strains. One potential target for rational drug design is the system responsible for purine salvage from the host environment, as trypanosomes are incapable of de novo synthesis of these essential compounds [1]. Whereas purine metabolism in trypanosomes has been extensively studied [1,2], relatively little is known about the uptake of purines, in particular purine nucleobases. Two high affinity transporters for adenosine, P1 and P2, have been identified in bloodstream forms of *T. b. brucei* and their substrate specificity has been partially characterised [3]. In addition, the P2 transporter has been shown to mediate the internalisation of melamino-arsenicals, including melarsoprol [3], an important drug in the treatment of late-stage sleeping sickness [4,5]. Moreover, the functional absence of P2 transport activity has been linked to melarsoprol resistance [3].

In contrast to purine nucleosides, little is known about the uptake of purine nucleobases by protozoan parasites. Only in a few protozoa have nucleobase transporters been characterised, and the mechanism of transport is unknown [6]. For bloodstream forms of *T.brucei*, preliminary data on the uptake of hypoxanthine was reported in 1980, but there have been no subsequent studies [7]. Recently, we have characterised a high affinity purine specific nucleobase transporter (H1) in the procyclic forms of *T. b. brucei* [8]. This transporter was selective for purine nucleobases and linked to protonmotive force, suggesting it functions as a nucleobase/proton symporter. In the current study we have investigated hypoxanthine uptake by bloodstream forms of *T. b. brucei* and characterised two additional nucleobase transporters, H2 and H3. The H2 transporter was inhibited by a broad range of both purine and pyrimidine bases as well as guanosine, and appeared to be linked to the protonmotive force. The H3 transporter, like the H1 carrier, was selective for purine bases.

2. Materials and methods

2.1. Trypanosomes

Trypanosomes (strain 427) from frozen stocks were grown in CD rats (Charles River) and separated from blood cells on a DE52 (Whatman) anion exchange column [9]. After collection they were counted in a haemocytometer, washed twice with the assay buffer (33 mM Hepes, 98 mM NaCl, 4.6 mM KCl, 0.55 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM NaH_2PO_4, 0.3 mM $MgCl_2$, 23 mM $NaHCO_3$ and 14 mM glucose, pH 7.3) and resuspended at 10^8 cells/ml. Previous studies [10] have shown that in bloodstream forms the membrane depolarises when the cells are prepared by the routine procedure of working at 4°C and storing them on ice before use. Moreover, this depolarization is often irreversible. To avoid this complication the trypanosomes were kept at 25°C at all times. At the end of each experiment, cell viability and motility were checked under a phase-contrast microscope.

2.2. Hypoxanthine transport assay

Hypoxanthine uptake was performed as described for procyclic trypanosomes [8]. Briefly, 100 μl of bloodstream forms of *T. b. brucei* in assay buffer (10^7 cells) were mixed with 100 μl of assay buffer containing [^{3}H]hypoxanthine (Amersham, 999 GBq/mmol) and, where appropriate, test compound. After predetermined times, normally 10 s, uptake was stopped by the addition of ice-cold buffer containing 4 mM of hypoxanthine, and centrifugation through an oil layer (7:1 v/v di-*n*-butyl phthalate/mineral oil). The resulting pellet was counted for accumulated radioactivity. Ionophores and ATPase inhibitors were usually preincubated with the cells for 3 min before addition of [^{3}H]hypoxanthine. The effect of com-

pounds dissolved in organic solvents was measured against controls containing the same percentage of solvent (ethanol or DMSO).

2.3. Intracellular pH and membrane potential

The intracellular pH (pH_i) and membrane potential (V_m) of bloodstream *T. b. brucei* were monitored and calibrated as described for procyclics [8], using the fluorescent dyes 2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF) and bis-(1,3-diethylthiobarbituric acid)trimethine oxonol (bisoxonol) (Molecular Probes), respectively and a Perkin-Elmer LS 50B fluorimeter. Cells were kept at 25°C throughout the experiment. pH_i was recorded after loading the trypanosomes for 1.5 h with 5 μM BCECF and washing twice with assay buffer, using $\approx 10^6$ cells per measurement (usually 5 min duration), during which the signal was strong and stable. Calibration in the presence of 20 μM nigericin was performed in low sodium, high potassium calibration medium (20 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 100 mM potassium gluconate, 10 mM glucose, 5 mM Mes and 5 mM Mops) as described [8]. Briefly, 30 μl of cells, kept at 25°C in calibration medium (pH 7.3) containing 20 μM nigericin, was added to 3 ml of calibration medium of various pH in a cuvette and the fluorescent intensity at 490 and 440 nm excitation (530 nm emission) was recorded for 2 min, the final value being the average of this recording (Fig. 3B).

For measurements of V_m, 10^7 cells in 100 μl assay buffer were added to 2.9 ml of 0.1 μM bisoxonol in assay buffer in a 3 ml cuvette. Test compounds (30 μl) were added from 100 × solutions, 60–90 s after addition of cells, to allow a stable recording of the resting potential. Identical control traces without cells were recorded and subtracted from the traces with cells, to correct for the effects of dilution and any fluorescence the test compound or solvent might induce. In addition, control traces substituting assay buffer or solvent for the test compound were recorded to correct for any instability of the signal during the recording time (4–10 min). Usually the signal was found to be stable during the recording, but in some cases corrections were made for a slight increase in fluorescence (apparent depolarization) during recording. Calibration was performed as described [8], using the method of Vieira et al. [11] (see also Fig. 3A). Briefly, gramicidin (5 μg ml^{-1}) was added to cells in potassium and sodium-free calibration buffer and K^+ concentration was increased stepwise. Under these circumstances, V_m approximates the electrochemical gradient for K^+ [12] and can be calculated from

$$V_m \approx E_{K^+} = -RT/F \ln [K^+]_i/[K^+]_o$$

in which V_m is the membrane potential, E_{K^+} is the K^+ equilibrium potential, $[K^+]_i$ and $[K^+]_o$ are the intracellular and extracellular concentrations of K^+, and R, T and F have their usual meanings. The value for $[K^+]_i$ was 116 mM [13].

2.4. Data analysis.

All experiments were carried out in triplicate unless otherwise stated. Errors given in Tables and Figs. are standard errors. In least-squares fits to the data, points were weighted according to the inverse of their relative SE. Statistical significance was determined using a two-sided unpaired Student's t-test. Inhibitor and kinetic constants (IC_{50} and K_m) were calculated from curves fitted using the Enzfitter and FigP computer programs (Elsevier Biosoft). K_i values were calculated from the Eq. $K_i = IC_{50}/(1 + (L/K_m))$, in which L is the permeant concentration, either 30 nM or 1 μM [^{3}H]hypoxanthine for H2 and H3 transporters, respectively. Protonmotive force was calculated from the equation $PMF = V_m - (2.3RT/F)(pH_i - pH_o)$ [14].

3. Results

3.1. Bloodstream forms of T. b. Brucei contain two purine nucleobase transporters

Time courses for the uptake of [^{3}H]hypoxanthine by *T. b. brucei* bloodstream forms at a hypoxanthine concentration of 30 nM and 1 μM showed that uptake was rapid and linear for at least 50 s (Fig. 1). The rate of uptake

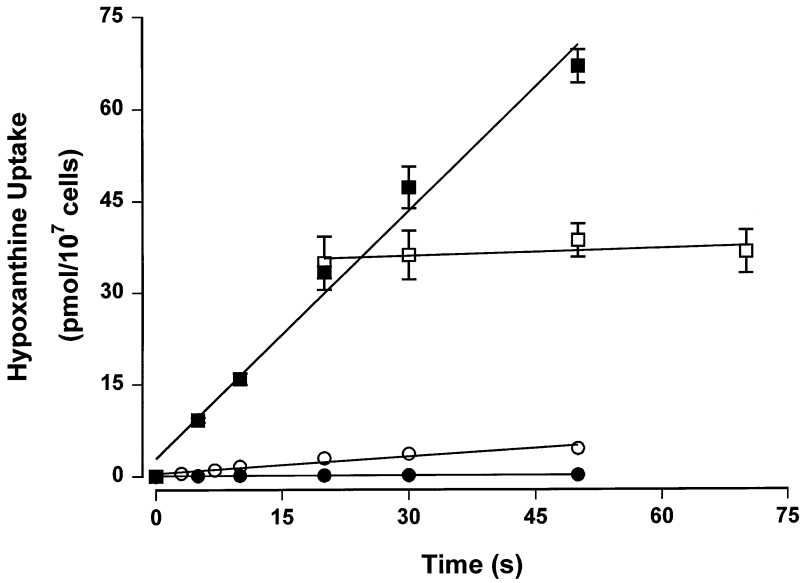

Fig. 1. Time course for [^{3}H]hypoxanthine uptake by bloodstream forms of *T. b. brucei*. Cells were incubated with [^{3}H]hypoxanthine at a final concentration of 30 nM (○) or 1 μM (■) and at the indicated times stopped by addition of 1 ml stop solution (ice-cold 4 mM hypoxanthine in assay buffer) and immediate centrifugation through oil. Non-mediated influx of hypoxanthine was assessed by determining the rate of 1 μM [^{3}H]hypoxanthine uptake in the presence of 1 mM unlabelled hypoxanthine (●). In an additional series (□), cells were incubated with 1 μM [^{3}H]hypoxanthine for 20 s, stopped with 1 ml stop solution and centrifuged after the indicated times (0, 10, 30, 50 s). The rate of uptake after addition of the stop solution was not significantly different from zero.

at 30 nM and 1 μM of [^{3}H]hypoxanthine was 0.09 ± 0.01 and 1.35 ± 0.08 pmol $(10^7$ cells$)^{-1}$ s^{-1}, respectively. The rate of influx of 1 μM [^{3}H]hypoxanthine in the presence of 1 mM hypoxanthine was 0.0016 pmol $(10^7$ cells$)^{-1}$ s^{-1}, indicating that non-mediated uptake accounts for less than 0.1% of internalised hypoxanthine at 1 μM.

Analysis of the transporter-mediated entry of metabolised permeants, such as hypoxanthine which can be converted to IMP by hypoxanthine phosphoribosyltransferase [1,2], requires methods to allow measurements of the initial rates of influx. Requirements for such methods are definitive time courses of permeant uptake (see Fig. 1) and a rapid means of separating cells from the extracellular radioactivity. In the present study uptake was terminated by addition of 1 ml ice-cold 4 mM hypoxanthine in assay buffer and centrifugation through an oil layer. To test the efficiency of this method of termination, stop solution was added to bloodstream forms of *T. b. brucei* incubated with 1 μM [^{3}H]hypoxanthine for 20 s and cells were centrifuged after 0 to 50 s. Fig. 1 shows that 4 mM hypoxanthine did effectively stop [^{3}H]hypoxanthine uptake. In all subsequent uptake assays initial rates of transport were determined at 10 s, well within the linear range.

At 1 μM [^{3}H]hypoxanthine, 1–2 mM guanosine inhibited a significant fraction ($\approx 90\%$) of hypoxanthine influx (Fig. 2). However, $11.4 \pm 1.9\%$ ($n = 8$) of the hypoxanthine influx was insensitive to guanosine inhibition, but was inhibited by hypoxanthine, showing the presence of at least two transporters for hypoxanthine in bloodstream forms of *T. b. brucei*. Uptake of [^{3}H]hypoxanthine by the guanosine-insensitive transporter, defined as hypoxanthine influx in the presence of 2 mM guanosine, was saturable and conformed to Michaelis-Menten kinetics (Fig. 2, inset), with an apparent K_m of 4.7 ± 0.9 μM and a V_{max} of

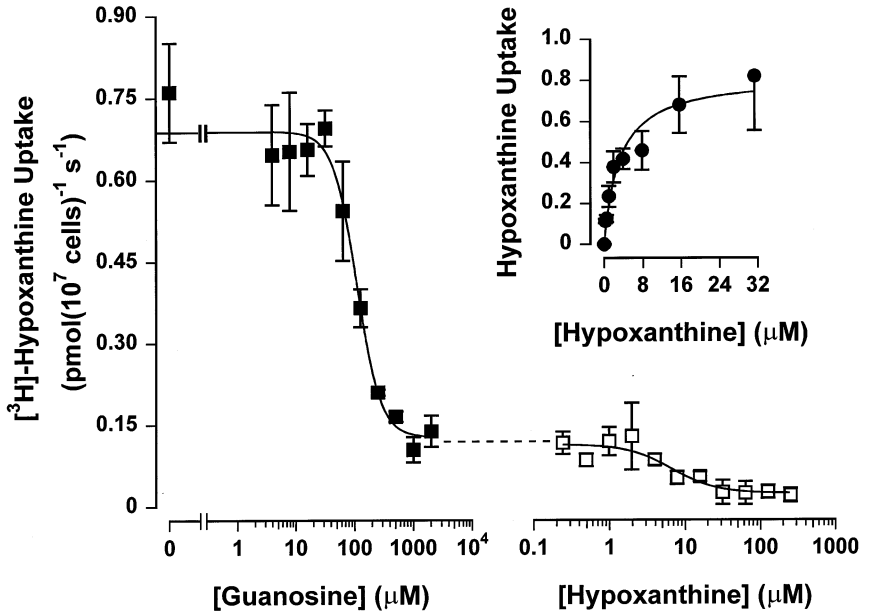

Fig. 2. Demonstration of guanosine-sensitive and -insensitive hypoxanthine transport in *T. b. brucei* bloodstream forms. Left panel: Initial rates of 1 μM [^{3}H]hypoxanthine influx were measured in the presence of increasing guanosine concentrations (■). IC_{50} for guanosine was 99 μM. At 2 mM of guanosine (maximal inhibition) the guanosine concentration was kept constant and unlabelled hypoxanthine was added (□, right panel), yielding further inhibition of [^{3}H]hypoxanthine influx. Conversion of the latter data to total hypoxanthine uptake (pmol $(10^7$ cells$)^{-1}$ s^{-1}) yielded a saturable curve (inset) and a K_m of 3.1 ± 0.9 μM and a V_{max} of 0.82 ± 0.07 pmol $(10^7$ cells$)^{-1}$ s^{-1}.

1.1 ± 0.1 pmol $(10^7$ cells$)^{-1}$ s^{-1} ($n = 3$). Hypoxanthine transport by the guanosine-sensitive hypoxanthine transporter was studied at 30 nM [^{3}H]hypoxanthine. At this concentration, $> 95\%$ of saturable hypoxanthine uptake was inhibited by guanosine (not shown) and the K_m of this component was found to be 123 ± 15 nM with a V_{max} of 1.1 ± 0.2 pmol $(10^7$ cells$)^{-1}$ s^{-1} ($n = 4$). The guanosine—sensitive and -insensitive—hypoxanthine transporters were designated H2 and H3, respectively.

Morphological examination of the trypanosomes stained with Giemsa, both before and after purification by anion-exchange, revealed that the population of trypanosomes used in this study was entirely of the long-slender form. Thus, it is unlikely that the presence of two distinct purine nucleobase transporters in bloodstream forms of *T. b. brucei* is due to the simultaneous existence of the different life-cycle forms, long-slender and short-stumpy.

3.2. Substrate specificity of the H2 and H3 transporters

The inhibitory effects of potential substrates for the hypoxanthine transporters were tested by measuring initial uptake rates at 30 nM (H2) and 1 μM [^{3}H]hypoxanthine (H3), in the presence of varying concentrations of the test compound. For the H3 transporter, incubations were performed in the presence of 1 or 2 mM of guanosine to block uptake by the H2 transporter. Results were plotted as log inhibitor concentration versus hypoxanthine uptake and, where applicable, K_i values and Hill slopes were calculated.

The results summarised in Table 1 suggest that the H2 transporter shows a relatively broad specificity, displaying highest affinity for purine nucleobases. The naturally occurring nucleobases adenine, guanine and xanthine inhibited hypoxanthine transport with K_i values between 0.3 and 10 μM. The purine nucleobase analogues allopurinol

Table 1
K_i values for potential inhibitors of hypoxanthine uptake in *T. b. brucei* bloodstream forms

Compound	H2 $K_i \pm$ SE (μM)	*n*	H3 $K_i \pm$ SE (μM)	*n*
Adenine	3.2 ± 1.1	3	8.8 ± 4.0	3
Guanine	0.36 ± 0.18	3	5.6 ± 2.2	2
Xanthine	8.8 ± 3.9	3	28.8 ± 1.7	3
Allopurinol	4.0 ± 2.2	3	194 ± 58	3
Oxypurinol	15.7 ± 3.5	3	>250	3
Thymine	82 ± 25	4	NE[a]	2
Cytosine	>500	3	NE[a]	2
Uracil	60 ± 14	3	NE[a]	2
Adenosine	590 ± 175	3	NE[a]	3
Inosine	167 ± 58	2	NE[a]	2
Guanosine	10.9 ± 1.8	4	NE[a]	6
Thymidine	>1000	2	NE[d]	2
Cytidine	NE[a]	2	NE[d]	2
Uridine	>500	3	NE[d]	2
Tubercidin	NE[a]	1	ND	
Dipyridamole	NE[c]	1	ND	
Dilazep	>50	2	ND	
Nitrobenzylthioinosine	NE[b]	1	ND	
Papaverine	NE[a]	1	ND	

The dose response curves were performed with a range of different inhibitor concentrations (at least six) and each data point performed in triplicate. Initial rates of 30 nM (H2) or 1 μM (H3) [^{3}H]hypoxanthine uptake were then plotted versus log inhibitor concentrations to determine IC_{50} values. From these, K_i values were calculated based on a K_m value of 123 nM and 4.7 μM for H2 and H3, respectively. Initial rates of [^{3}H]hypoxanthine influx via the H3 transporter were determined in the presence of 1 mM guanosine. NE, no effect, meaning $<10\%$ stimulation or inhibition at [a] 1 mM, [b] 10, [c] 50 or [d] 250 μM of test compound; ND, not determined; *n*, number of separate experiments.

and oxypurinol also inhibited transport with high affinity (4.0 ± 2.2 and 15.7 ± 3.5 μM, respectively). In addition, the purine nucleoside guanosine strongly inhibited [^{3}H]hypoxanthine uptake ($K_i = 10.9 \pm 1.8$ μM), but none of the other nucleosides tested had a significant effect at concentrations less than 100 μM. Similarly, hypoxanthine uptake was unaffected by nitrobenzylthioinosine, dipyridamole, dilazep and papaverine, all potent inhibitors of some of the mammalian purine transporters [15–17]. However, the pyrimidine nucleobases uracil and thymine inhibited hypoxanthine transport with medium affinity (K_i values between 50 and 100 μM). Hill slopes were generally found to be near -1 and inhibition by these compounds reached near 100% inhibition, showing that, at 30 nM [^{3}H]hypoxanthine, selective uptake by a single transporter was being assessed.

Compared to the H2 transporter, the H3 carrier displayed a much narrower specificity, being insensitive to any nucleosides or pyrimidine nucleobases up to millimolar range (Table 1). Allopurinol and oxypurinol were less potent inhibitors of hypoxanthine influx mediated by the H3 transport system. Only the natural purine nucleobases inhibited [^{3}H]hypoxanthine transport by this system, with K_i values from 5.6 to 28.8 μM.

3.3. Ionophores inhibit [^{3}H]hypoxanthine transport by the H2 transporter

Active nucleoside transport in mammals is dependent on the sodium gradient over the plasma membrane [15,16]. There has also been a report that glucose transport in bloodstream form typomastigotes is sodium-dependent [18], but most workers have dismissed this report and concluded that glucose transport is independent of any ions [19–21]. Similarly controversy surrounds the possibility of proton-dependent uptake of metabolites

in bloodstream and procyclic forms of typomastigotes and other trypanosomatid parasites [21–23]. We previously suggested that the H1 nucleobase transporter in *T.b.brucei* procyclics is proton-driven [8]. The possibility that H2-mediated [^{3}H]hypoxanthine in *T. b. brucei* bloodstream forms might similarly be dependent on the protonmotive force (PMF) rather than the sodium gradient was tested using ionophores. Table 2 demonstrates that whereas the sodium ionophore monensin had no effect on hypoxanthine uptake, several other ionophores inhibited this process in a dose-dependent manner. These include the proton ionophore carbonyl cyanide chlorophenylhydrazone (CCCP), the K^+/H^+ exchanger nigericin, the K^+ ionophore valinomycin and the Na^+/K^+ ionophore gramicidin. These agents all affect the electrochemical gradient of either protons or potassium (or both) over the plasma membrane and their effect on hypoxanthine transport is evidence for active transport and an indication for a dependence on PMF. This finding was also supported by the observation that *N*,*N*′-dicyclohexylcarbodiimide (DCCD) and *N*-ethylmaleimide (NEM), compounds that inhibit the *T. b. brucei* [10], *T. cruzi* [24], and *Leishmania major* plasma membrane proton pumps [11,25], blocked hypoxanthine influx.

Table 2
Effects of ionophores and ATPase inhibitors on H2-mediated [^{3}H]hypoxanthine uptake by *T. b. brucei* bloodstream forms

Compound	[Compound] (μM)	Transport (% of control)	*P*
CCCP	1	60.9 ± 4.6	<0.01
	5	49.8 ± 4.0	<0.001
	20	44.6 ± 5.6	<0.001
Nigericin	1	105 ± 6.7	NS
	10	77.7 ± 4.9	<0.05
	20	50.7 ± 4.5	<0.001
	50	34.0 ± 4.4	<0.001
Valinomycin	0.3	98.8 ± 9.8	NS
	1	71.2 ± 9.8	NS
	5	57.9 ± 6.8	<0.02
	10	48.2 ± 3.6	<0.001
Monensin	10	114 ± 6.9	NS
	25	87.7 ± 6.8	NS
NEM	200	85.6 ± 7.4	NS
	1000	56.0 ± 5.0	<0.001
Gramicidin	1	77.2 ± 9.2	NS
	5	49.7 ± 1.4	<0.01
	10	28.1 ± 2.2	<0.001
	25	24.6 ± 2.6	<0.001
DCCD	10	94.0 ± 8.6	NS
	100	31.5 ± 2.7	<0.001
	500	13.9 ± 2.0	<0.001

Cells were preincubated for 2 min (CCCP) or 3 min (all other compounds) and the initial rate of 30 nM [^{3}H]hypoxanthine influx was measured. Data shown are the average of six measurements $\pm$ SE. Uptake was statistically tested against a control run in parallel which was receiving the same treatment but was solvent instead of compound.

3.4. The H2 transporter is dependent on the protonmotive force

To further investigate whether hypoxanthine transport is driven by PMF, changes in PMF resulting from the actions of the ionophores and proton pump inhibitors were quantified. Quantitative measurements of V_m and pH_i were determined, using the fluorescent probes bisoxonol and BCECF, respectively. Calibration of bisoxonol fluorescence by stepwise increases of potassium in the presence of gramicidin [11], established a linear range of about -125 to -25 mV (Fig. 3A). At concentrations of potassium corresponding to higher voltages no further increase in fluorescence was observed, a finding similar to that obtained by Defrise-Quertain et al. [10]. Thus, bloodstream forms of *T. b. brucei* seem able to compensate for very high concentrations of extracellular potassium. While this limits the range in which bisoxonol fluorescence can be converted to V_m, the conversion is still valid in the linear range. The resting potential of *T. b. brucei* bloodstream forms was found to be -102.5 ± 1.2 mV ($n = 38$) at 25°C.

Calibration of BCECF-monitored pH_i was carried out in the presence of 20 μM nigericin and yielded a straight line from pH 6.0 to pH 8.0 (Fig. 3B). Under standard conditions (25°C, pH 7.3), pH_i was 7.18 ± 0.01 ($n = 30$). The pH_i was stable at extracellular pH (pH_o) 7.0–8.0, but dropped to 6.64 ± 0.01 at pH_o 6.5.

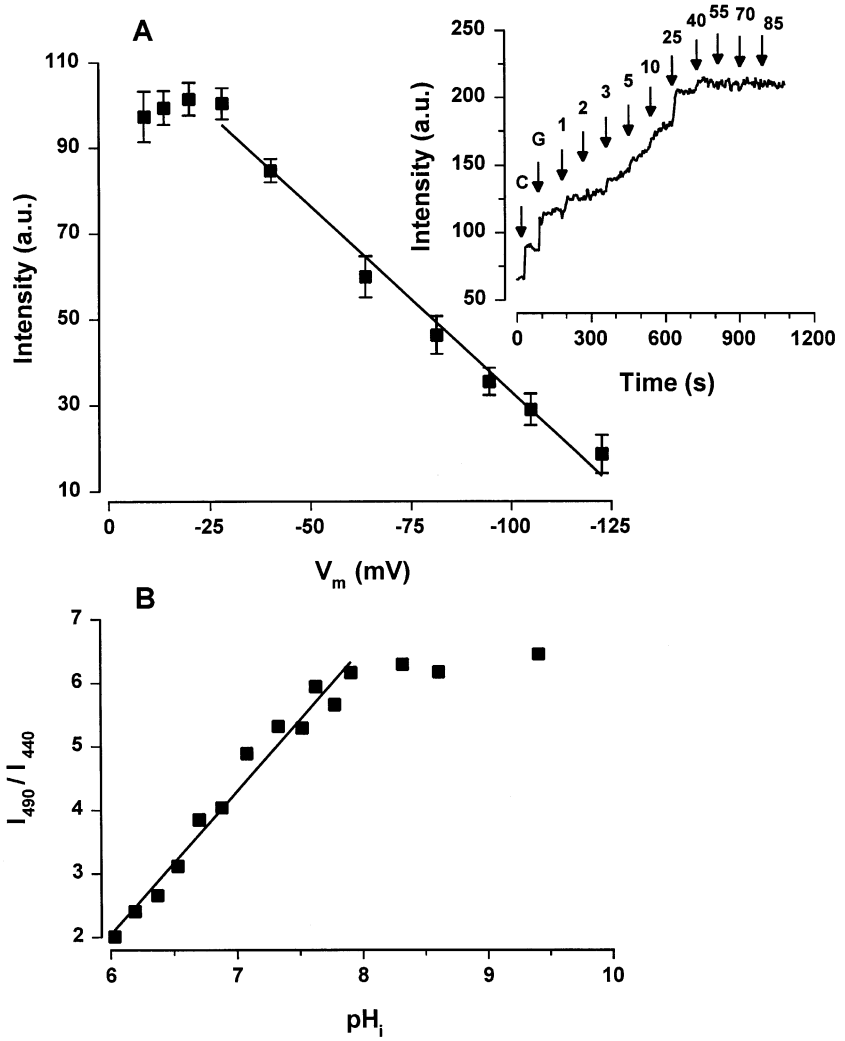

Fig. 3. Calibration of membrane potential and intracellular pH. (A) Fluorescent intensity of *T. b. brucei* bloodstream forms in calibration buffer was monitored in the presence of 0.1 μM bisoxonol (inset). At 60 s, cells (C) were added followed by gramicidin (G, 5 μg/ml) at 120 s. At the indicated times (90 s apart), KCl in calibration buffer was added to the final concentrations indicated (mM). Average fluorescence during the last 30 s before the next addition was plotted against the calculated value of V_m. (B) The ratio of fluorescence intensity at 490 nm (I_{490}) and 440 nm (I_{440}) in the presence of nigericin was determined for *T. b. brucei* bloodstream forms in calibration medium of various pH. The resulting standard curve shows a linear range from pH 6.0 to 8.0.

V_m and pH_i were monitored for 3–5 min after test compound was added to the cuvette (Fig. 4 and Fig. 5). CCCP or nigericin, at 20 μM, both induced rapid, strong acidification in *T. b. brucei* bloodstream forms, with a relatively small effect on V_m ($\approx +25$ mV). In contrast, 100 μM DCCD almost completely depolarised the cells. A smaller, but significant effect on pH_i was also observed. Gramicidin (10 μM) had no effect on pH_i, but depolarised the cells even more rapidly than DCCD (not shown). To quantify these results, the effect of a compound was taken to be the average V_m or pH_i during the last 1–2 min of recording, corresponding to the 3 min preincubation in the transport assay. The results of these measurements were used to calculate the PMF in the

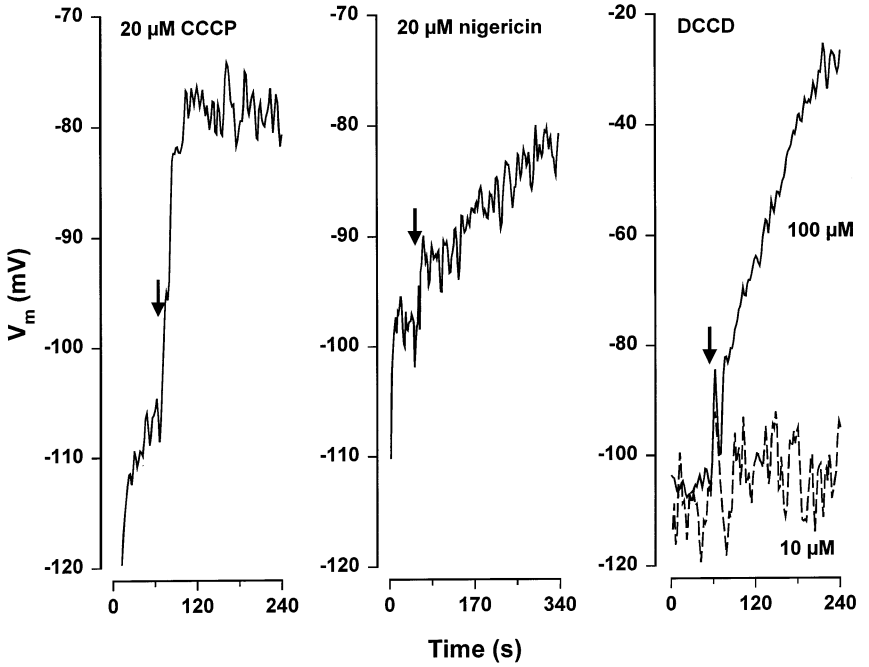

Fig. 4. Effects of CCCP, nigericin and DCCD on membrane potential. Fluorescent intensity of bisoxonol in the presence of 10^7 *T. b. brucei* bloodstream forms, added at $t = 0$, was recorded. At the indicated times (arrow), test compounds were added to the cuvette to the final concentrations shown in the figure. Traces shown are representative of three to eight experiments.

presence and absence of the various compounds (Table 3). This in turn was plotted against the rate of hypoxanthine uptake as shown in Table 2 and yielded a straight line with a correlation coefficient of 0.95 (Fig. 6). No correlation was observed between rate of hypoxanthine tranport versus pH_i ($r^2 = 0.07$), whereas the correlation between hypoxanthine influx versus V_m ($r^2 = 0.71$) was markedly less than that shown between transport activity and PMF.

These results provide strong evidence for a coupling of hypoxanthine uptake to the PMF and suggest the H2 hypoxanthine transporter could be a nucleobase/H^+ symporter. As hypoxanthine is neutral under standard conditions ($pK_a = 5.3$), symport with protons should be electrogenic and induce membrane depolarisation. However, hypoxanthine by itself had little effect on V_m (Fig. 7). Only after treatment with NEM, designed to inhibit the plasma membrane proton-pump, did hypoxanthine induce a gradual, pronounced depolarisation. The average V_m 7–8 min after the addition of hypoxanthine (added 1 min after NEM) was -51.0 ± 3.0 mV ($n = 4$) as opposed to -74.2 ± 0.8 mV ($n = 3$) after adding assay buffer ($P < 0.01$).

4. Discussion

The present study establishes the existence of two distinct nucleobase transporters (H2 and H3) in *T. b. brucei* bloodstream forms. Both were shown to be saturable carriers with the H2 carrier displaying a 40-fold higher affinity for hypoxanthine (123 ± 15 nM and 4.7 ± 0.9 μM for the H2 and H3 transporters, respectively). In addition, the two transporters differed in their K_i values for a range of potential permeants, with the H2 carrier generally displaying the higher affinity, most notably for guanosine and the pyrimidine nucleobases uracil and thymine. In both cases, however, the naturally occurring purine bases hypoxanthine, adenine, guanine and xanthine

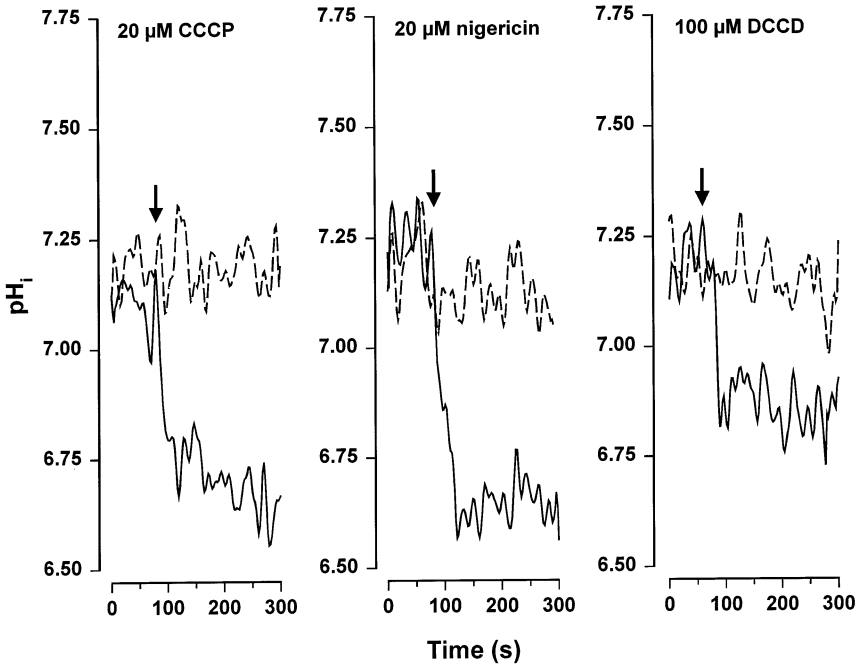

Fig. 5. Effects of CCCP, nigericin and DCCD on pH_i. Fluorescent intensity (I_{490}/I_{440}) of $\approx 10^6$ bloodstream *T. b.* brucei was recorded. At the indicated times (arrow), either the test compound or the same volume of ethanol (solvent in all three cases; dashed line) was added to the cuvette. Traces shown are representative of three to six similar experiments.

seemed to be the main substrates, perhaps reflecting the dependency of protozoa on purine salvage [1]. These purine nucleobase transporters are distinct from the purine nucleoside transporters P1 and P2 reported for bloodstream forms of *T. b. brucei* [3] as they have no affinity for adenosine (K_i values >500 μM). It thus appears that bloodstream *T. b. brucei* have at least four distinct high affinity purine carriers. The existence of multiple independent uptake systems has clear implications for the development of new chemotherapy aimed at blocking purine salvage, as no single purine carrier seems to be essential. In addition, the present study may also be of importance to our understanding of drug resistance. For example, resistance of *T. b. brucei* to melarsoprol has been linked to the loss of the P2 transporter [3]. Further studies will be required to determine whether the H2 and H3 purine nucleobase transporters are also involved in the transport of existing trypanocidal drugs.

The presence of multiple purine transporters in bloodstream forms of *T. b. brucei* is in contrast with the simpler situation in procyclic forms, which have only a single purine nucleobase transporter [8] and one purine nucleoside transporter (unpublished observation). The procyclic nucleobase transporter (H1) is similar to the H3 carrier described here. Indeed, the K_m for hypoxanthine of the H1 carrier (9.3 ± 2.0 μM) is not significantly different from the K_m of the H3 transporter and both transport systems are selective for purine nucleobases only, interacting with all naturally occurring purine bases with high affinity. However, there are some marked differences between the two systems, notably the 4-fold higher K_i value for xanthine of the H3 transporter compared to the H1 transporter (28.8 ± 1.7 vs 7.2 ± 0.6 μM; $P < 0.01$) and the loss of high affinity for the purine analogue allopurinol (K_i values of 194 ± 58 and 5.0 ± 0.9 μM for H3 and H1, respectively). The question of whether the H1 and H3 transporters are actually the same carrier,

Table 3
Effect of ionophores and ATPase inhibitors on protonmotive force across plasma membranes of bloodstream *T. b. brucei*

Compound	[Compound] (μM)	pH_i	V_m (mV)	PMF (mV)
None	—	7.18 ± 0.02	-102.5 ± 1.2	-95.4 ± 1.3
CCCP	20	6.74 ± 0.03	-78.9 ± 1.6	-45.6 ± 2.4
DCCD	10	7.21 ± 0.02	-103.9 ± 0.6	-98.5 ± 1.0
	100	6.98 ± 0.01	-32.9 ± 5.8	-14.0 ± 5.8
Gramicidin	10	7.18 ± 0.04	-20[a]	-13.1 ± 2.8
Nigericin	20	6.70 ± 0.03	-82.5 ± 1.0	-46.9 ± 1.8
NEM	200	6.83 ± 0.08	-101.1 ± 2.6	-73.2 ± 5.1
	1000	6.35 ± 0.08	-95.3 ± 0.8	-39.3 ± 4.6

Data are the average of three to six recordings. The effect of the compounds on pH_i or V_m are taken as the average value determined 3 min after the addition of the test compound (see Fig. 4 and Fig. 5).
[a] The standard curve for V_m allows accurate conversion of fluorescence up to -20 mV. Gramicidin induced a rapid increase in fluorescence, reaching values outside this range within 2 min. A value of -20 mV has been substituted for the actual value and the value given for the PMF is therefore an estimate.

perhaps modified in the different life-cycle stages, awaits further studies at the molecular level. We also do not know if the H3 transporter functions as a proton-dependent system as has been suggested for the H1 carrier in procyclic cells [8].

The high affinity and selectivity for purine nucleobases of the H3 and H1 transporters separates them from nucleobase transporters so far reported in other protozoan species. Nucleobase transporters in *Giardia intestinalis* [26] and *Tritrichomonas foetus* [27], for example, display permeant affinities in the millimolar range and a hypoxanthine transporter in *Plasmodium berghei* also transports adenosine and inosine [28]. None of these transporters is similar to the *T. b. brucei* H2 transporter either, though the *G. intestinalis* carrier also transports uracil and thymine along with hypoxanthine and adenine [26]. However, a possible similar carrier for hypoxanthine and adenine has been reported for the related kinetoplast *Leishmania braziliensis panamensis* [29], but the affinity for adenine (30 μM) was an order of magnitude lower than for any of the *T. b. brucei* carriers and no studies have been reported on whether this transporter accepts other purine or pyrimidine bases as permeants. The H2 transporter was also distinct from any known mammalian nucleobase transporters, as it was insensitive to inhibition by papaverine, dipyridamole and dilazep [17,30].

The clear effects of various ionophores on H2-mediated hypoxanthine transport by *T. b. brucei* bloodstream forms indicates active transport rather than facilitated diffusion as the uptake mechanism. While active nucleobase transport in mammals is through Na^+-dependent carriers [15,17] there have been few reports on the existence of active transport in bloodstream forms of typomastigotes. Sodium-dependent glucose uptake by bloodstream forms of *T. b. rhodesiense* has been reported [18], but most workers believe the system is catalysed by a facilitiated-diffusion system [19–21]. The possibility of proton-dependent uptake systems in bloodstream forms of trypanosomes has also been considered to be an unlikely option based on ΔpH across the membrane [13]. In other trypanosomatids, including *Leishmania*, the existence of proton-dependent uptake systems has been based mainly on indirect evidence with the exception of direct eletrophysiological measurements of the *Leisamania donovani myo*-inositol transporter expressed in oocytes [21–23,31]. Given this controversy surrounding proton-dependent uptake of metabolites by trypanosomatids, we undertook a detailed investigation of the role of protonmotive force in the uptake of hypoxanthine via the H2 system.

Under standard experimental conditions (25°C, pH 7.3), *T. b. brucei* bloodstream forms were found to have a near-neutral pH (7.18 ± 0.01) and a highly negative V_m (-102 ± 1 mV). The maintenance of near neutral pH in bloodstream forms has been well documented [32,33], but the V_m seems to depend strongly on temperature, pH and buffer conditions [32–34]. Estimates range from -155 mV at 22°C [32], -82 mV at 30°C [34] to -40 mV at 37°C. Our estimate of the resting V_m of -102 mV at 25°C is in accordance with these earlier studies for bloodstream forms of *T. b. brucei* and is close to reported values of -110 mV in *T. cruzi* epimastigotes [24] and -94 mV for *T. b. brucei* procyclics [8].

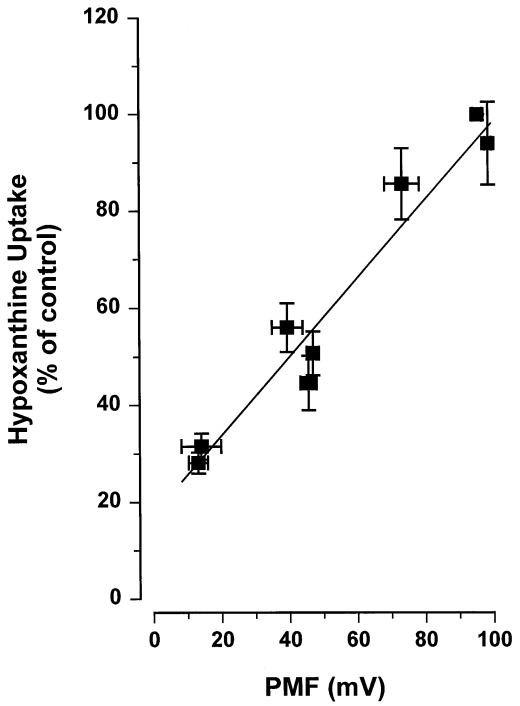

Fig. 6. Linear correlation between [^{3}H]hypoxanthine uptake and PMF. Initial rates of 30 nM [^{3}H]hypoxanthine in the presence and absence of ionophores and ATPase inhibitors, as listed in Table 2, were plotted against the effects of these compounds on PMF, as listed in Table 3. Linear regression using relative weighting of data points yielded a line with a correlation coefficient of 0.95, a slope of 0.81 ± 0.18 and a y-axis intercept of 17.5 ± 8.6 (not significantly different from zero).

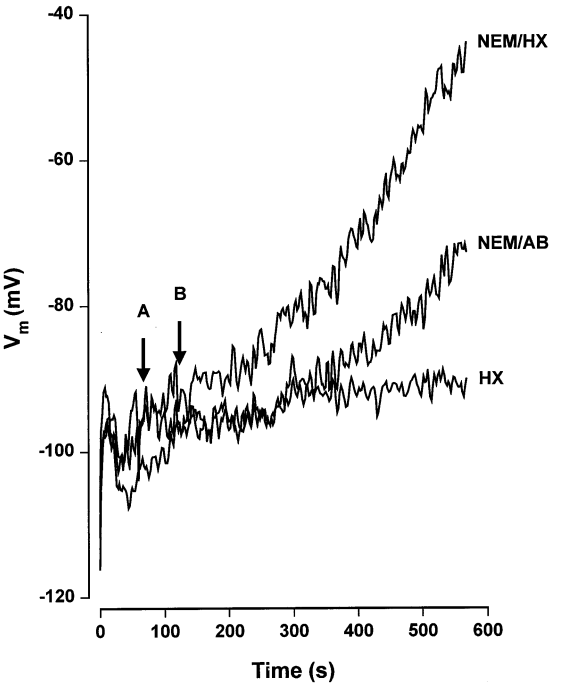

Fig. 7. Effect of hypoxanthine on membrane potential of *T. b. brucei* bloodstream forms. Fluorescence of bloodstream forms in the presence of bisoxonol was recorded. At $t = 0$ s, cells were added, and at arrow A either NEM (1 mM final concentration) or hypoxanthine (HX, 40 μM; lower trace) was added. At arrow B, hypoxanthine (40 μM) or assay buffer (AB) was added to the top and middle traces, respectively. Traces shown are representative of four to five similar experiments.

Given the strong dependence of V_m on experimental conditions, it was essential for the investigation of the effects of PMF on hypoxanthine transport that we conduct our own quantitative measurement of V_m, under the conditions that were used in the transport assays. While calibration of V_m was hampered by the presence of a mechanism compensating for high concentrations of K^+ [10], limiting the operational range for bisoxonol in these cells (-125 to -25 mV), judicious use of inhibitor concentrations allowed conditions to be selected that produced effects on V_m within the standard curve.

In *T. b. brucei* bloodstream forms, the sodium ionophore monensin had no effect on hypoxanthine transport, but H^+-ATPase inhibitors (DCCD and NEM), ionophores for H^+ (CCCP)

and K^+ (valinomycin) as well as K^+/Na^+ (gramicidin) and K^+/H^+ (nigericin) exchangers, dose-dependently inhibited hypoxanthine uptake. Each of these inhibitors was shown to reduce the protonmotive force, principally by either cytosolic acidification (nigericin, CCCP), by plasma membrane depolarisation (gramicidin) or both (DCCD). The fact that either pH_i or V_m might remain unchanged by an inhibitor of hypoxanthine transport, as long as one of the two parameters is sufficiently affected, clearly indicates that transport rates are not determined by cytosolic pH or by the membrane potential per se, but rather by the protonmotive force, which is composed of both parameters. The demonstration of a linear correlation between hypoxanthine uptake and protonmotive force (Fig. 6) strongly supports this conclusion.

A model for the uptake of hypoxanthine through a nucleobase/proton symporter would predict that the uptake be electrogenic. This criterion was fulfilled by the observation that hypoxanthine induced a marked membrane depolarisation in the presence of NEM (added to block the H^+-ATPase). In the absence of NEM, it is proposed that the proton pump compensates for the influx of protons associated with hypoxanthine transport.

In conclusion, the current study establishes the presence of two distinct nucleobase transporters in bloodstream forms of *T. b. brucei*, with different affinities and specificities. Hypoxanthine transport through at least one of these is coupled to the proton electrochemical gradient, consistent with it being a nucleobase/proton symporter.

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