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PENTAMIDINE TRANSPORT IN *TRYPANOSOMA BRUCEI*—KINETICS AND SPECIFICITY*

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Abstract—Pentamidine, an aromatic diamidine used in the treatment and control of African trypanosomiases, is rapidly absorbed by *Trypanosoma brucei* and is lethal to the parasite both *in vitro* and *in vivo*. The pentamidine transport system in *T. brucei* exhibits saturation kinetics and shows specificity for the aromatic amidine moiety of this compound. The inhibition of uptake by salicyl hydroxamic acid (SHAM) and iodoacetate (IAA), and the retention of intracellular substrate against large chemical gradients indicate a coupling of energy to this system. The system shows high affinity for pentamidine with an average K_m value of 2·68 μM. Structural specificity in relationship to varying drug affinities for this transport site and the role it might play in drug resistance are discussed.

Pentamidine (Fig. 1) is an aromatic diamidine used in the treatment and control of African trypanosomiases. The exact mechanism of drug action, as well as the basis for its parasite selectiveness over host cells, is unknown. It is known that pentamidine inhibits various metabolic processes in a variety of organisms. Low concentrations of the drug halt cell growth of *Escherichia coli* K12 [1], *Staphylococcus aureus* [2] and *Leptomonas* sp. [3]; inhibit DNA, RNA and protein synthesis in 6H3 HED ascites tumor cells [4] and *S. aureus* [2]; interfere with amino acid accumulation in *S. aureus* and oxygen consumption in *Crithidia fasiculata* [5].

Trypanocidal compounds have been divided into two main groups based on their biological activity [6]. One group inhibits infectivity, without rapidly killing the organisms. This group of drugs is not rapidly concentrated in large amounts. Pentamidine falls into the second group of drugs, which rapidly kills the trypanosomes *in vivo* and *in vitro*, fails to inhibit infectivity of viable drug-exposed trypanosomes and is concentrated by the organisms in large amounts. The present study was carried out in order to determine whether blood trypomastigote forms of *Trypanosoma brucei* have a pentamidine transport system and, if so, to analyze its kinetics and substrate specificity.

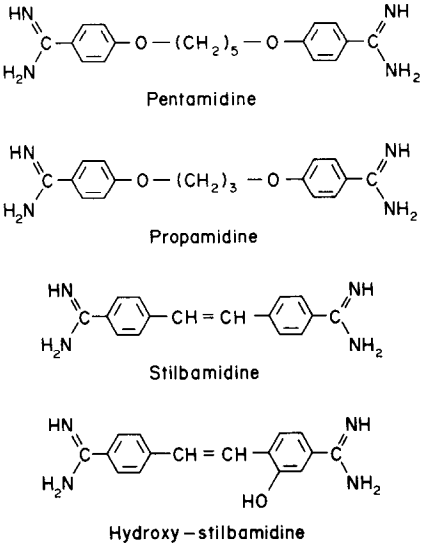

Fig. 1. Structures of trypanocidal aromatic diamidines.

MATERIALS AND METHODS

Isolation of organisms. Trypanosomes were routinely isolated from female Sprague–Dawley rats infected with a highly virulent, monomorphic substrain of the EATRO laboratory strain 110 M of *T. brucei*. The history of the parent strain and substrain, as well as methods used in isolating, purifying and preserving frozen stock, has been described elsewhere [7,8]. Rats were bled by cardiac puncture and the blood was defibrinated with glass beads. Trypanosomes were separated from blood cells by differential centrifugation for 15 min at 5° at 1000 *g* (swinging bucket, PR1 International centrifuge). Serum with buffy coat was removed, and rabbit anti-rat blood cell antibody was added to it. After incubation with the antibody at room temperature for 15 min, the trypanosome suspension was repeatedly centrifuged to remove any contaminating blood cells and platelets as determined by microscopic examination. Throughout the isolation procedure the parasites were kept cold and in host serum. An aliquot of the suspension was diluted in a Unopette blood-diluting pipette (Becton & Dickinson), and the cells were counted in the improved Neubauer hemocytometer. Immediately before being

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used in uptake studies, cells were centrifuged for 1 min at 12,000 *g* at 5° in a Sorvall Superspeed RC 2B centrifuge. The supernatant was discarded and the trypanosomes were resuspended in Hanks' balanced salt solution containing 100 mg/100 ml glucose and 50 mg/100 ml albumin (HBSSA) to give a final concentration of 2×10^8 cells/ml.

Chemical compounds. Propamidine, stilbamidine, 2-hydroxystilbamidine and pentamidine were graciously provided by May & Baker, Ltd. (Dagenham, England). L-Arginine, L-lysine, *para*-aminobenzoic acid (PABA) and salicyl hydroxamic acid (SHAM) were purchased from Calbiochem, and [^{14}C]inulin (sp. act., 2·56 mCi/g) was purchased from New England Nuclear. Benzamidine was kindly provided by Dr. Yale Nemerson.

Preparation and isolation of [^{3}H]pentamidine. [^{3}H]pentamidine was prepared from 95 mg of the free base by catalytic exchange with tritiated water (25 Ci) over Rh/Al_2O_3 in acetic acid by New England Nuclear (Boston, Mass.). The radiolabeled compound, free from unincorporated tritium, was precipitated with 1 N NaOH, filtered and redissolved in warm 95% ethanol. Methane sulfonic acid was added to the solution (2 m-moles acid/m-mole of pentamidine base) and allowed to react in the cold overnight. The resulting [^{3}H]pentamidine methane sulfonate was filtered out of solution and its purity determined by comparison with a standard (methane sulfonate salt of pentamidine prepared from the free base in this laboratory) according to the following criteria: (1) melting point, which was 257° for the tritiated drug and 259° for the standard; (2) infrared spectrum, which showed characteristic peaks for the $-NH_2$, $-NH$, $-CH_2CH_2$ and phenol groups at 3320, 3120, 2950 and 1620 wavelength/cm respectively; and (3) migration behavior upon thin-layer chromatography (t.l.c.) on Silica gel (solvent system: butanol–formic acid–water, 7·5:1·5:1·0), in which the tritiated compound co-migrated with the standard. The specific activity of the tritiated drug was 1200 mCi/m-mole.

Protocol for uptake experiments. Solutions of HBSSA containing twice the final concentrations of [^{3}H]pentamidine and, where appropriate, inhibitors were thermally equilibrated in a 37° water bath. Washed trypanosomes (2×10^8 cells/ml) in HBSSA were pipetted into 50-ml Erlenmeyer flasks, each containing a 150-mm stirring bar, and were incubated in the water bath for 45 sec. Then a volume of [^{3}H]pentamidine equal to the volume of the cell suspension was pipetted into the flask and mixed on a magnetic stirrer. An electric timer (Lab-Chron Timer, Lab-line Instruments, Inc.) was activated at the time the drug was added.

At 10- to 15-sec intervals, 250-μl aliquots of this suspension were removed with an Eppendorf automatic pipette and layered on top of 50 μl silicone (G.E. versilube F-50; viscosity, 75 centistokes; sp. gr. 1·05), which was itself layered over 100 μl of 12% (v/v) perchloric acid (PCA) in a 500-μl plastic microfuge tube. The organisms were spun from the radiolabeled exposing solution through the silicone into the PCA layer by centrifugation for 1 min at 7000 *g* in a Beckman 152 Microfuge. Upon reaching the PCA, cells are lysed, releasing their soluble contents, and PCA-precipitable material is pelleted.

For counting, 10 μl of exposing solution (top layer) or 50 μl of the PCA-soluble fraction was added to 4 ml of a solution consisting of 1 g bis-MSB [*p*-bis-(*O*-methylstyryl)-benzene] and 7 g PPO (2,5-diphenyloxazole) dissolved in 340 ml Triton X-100 and 660 ml xylene. Samples were counted in a Beckman LS 250 liquid scintillation counter.

In analyzing the results, a correction was made for extracellular solution which passes through the silicone when trypanosomes are spun into the PCA layer. The volume of extracellular solution involved was determined by labeling the exposing solution with [^{14}C]inulin ($3·675 \times 10^5$ cpm/ml). Equal volumes of cell suspension and [^{14}C]inulin solution were mixed, then 250 μl was centrifuged and sampled as described under the protocol for pentamidine uptake. Samples taken from the PCA-soluble layer and counted showed no increase in radioactivity during a 5-min incubation and there was no rate of disappearance of ^{14}C from the top layer. This was interpreted to mean that the organisms were excluding [^{14}C]inulin and that the radioactivity present in the PCA-soluble layer was due to extracellular [^{14}C]inulin brought down with the cells. From the activity (cpm) found in the PCA-soluble layer, the volume of external solution brought down with the cells was calculated. The number of contaminating cpm represented by this volume for solutions used in transport studies was subtracted from the total cpm/sample.

Protocol for counterflow experiments. Trypanosomes were loaded with [^{3}H]pentamidine for 2 min. Four ml of the cell suspension was centrifuged at 12,000 *g* for 1 min and the supernatant discarded. Cells were then resuspended in 4 ml HBSSA. Two 250-μl samples of this suspension were taken for assay of initial intracellular radioactivity. Next, 2 ml of suspension was removed and added to a flask containing 10 μl of a solution of either pentamidine or hydroxystilbamidine. The remaining 1·5 ml of suspension served as control. Samples were taken alternately from the control and experimental flasks.

RESULTS

It was found that cells isolated in the manner described above, kept in host serum and on ice, maintain specific transport properties for at least 45 min. After removing the organisms from serum and resuspending them in HBSSA, transport rates were unaffected for as long as 20 min.

Since pentamidine is a trypanocidal agent, it was necessary to determine concentrations in which the cells remain viable for the length of time a given experiment was being carried out. The highest external concentration of pentamidine used in studying pentamidine transport was 0·1 mM. This is below the lethal concentration *in vitro* for these trypanosomes at 37° for 5 min. At concentrations as high as 1·0 mM, motility, infectivity, O_2 consumption and glucose uptake are not impaired over a period of 5 min [9].

The chemical purity of the [^{3}H]pentamidine used in this investigation was determined as described in Materials and Methods. The biological assay for chemical purity of the labeled compound was to evaluate its activity as a transport substrate in our transport studies. The rate of uptake was found to

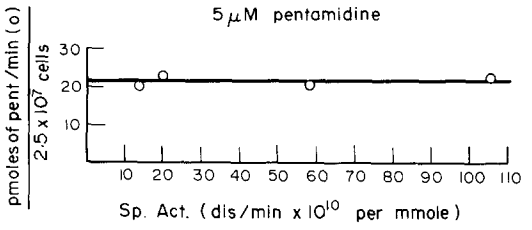

Fig. 2. Rate of pentamidine uptake by *T. brucei* at various specific activities (sp. act.. 14×10^{10} dis./min/m-mole, 20×10^{10} dis./min/m-mole, 58×10^{10} dis./min/m-mole and 106×10^{10} dis./min/m-mole).

be independent of specific activity over a range of 1·4 to 11·0 $\times 10^{11}$ dis./min/m-mole (Fig. 2).

Concentration dependence of pentamidine uptake. In a series of 16 experiments, the initial rates of [^{3}H]pentamidine uptake at various concentrations were determined (Fig. 3). A range of K_m values between 1·5 and 3·1 μM was obtained. A typical saturation curve and Lineweaver–Burk plot are shown in Fig. 4A and 4B. Statistical analysis of the data from 16 experiments gives a mean value of 2·68 μM for the K_m with a standard deviation of 0·42. A mean value of 91 pmoles/min/2·5 $\times 10^7$ cells is obtained for the V_{max} value with a standard deviation of 0·52. A plot of the intracellular concentration of pentamidine versus the external concentration is shown in Fig. 4B (insert). At 1 min, the [pent]$_i$ is consistently higher but varies with [pent]$_e$ with ratios ranging from 2·3 times higher at 4·5 μM up to 17 times higher at 0·34 μM. Higher ratios are attained after longer periods

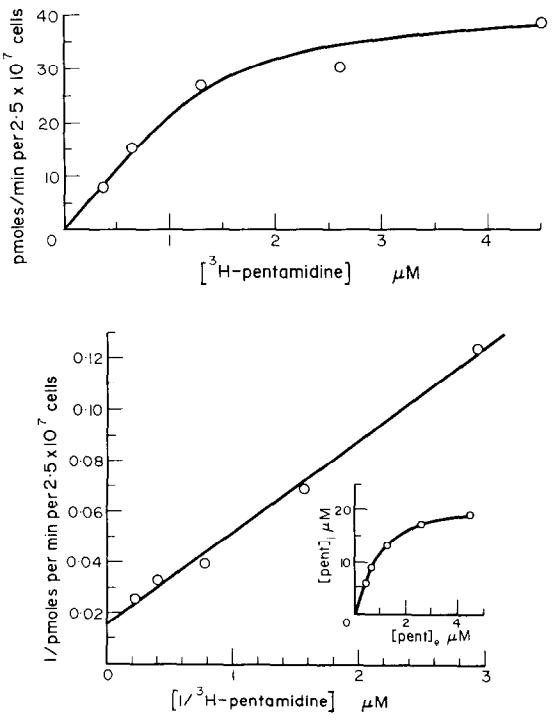

Fig. 4. (A) A plot of initial rates of transfer against external pentamidine concentration. (B) Double reciprocal plot of uptake data shown in A. The K_m value is 2·3 μM and the V_{max} value is 63 pmoles/min/2·5 $\times 10^7$ cells. Insert: plot of intracellular concentrations, [pent]$_i$, against extracellular concentrations, [pent]$_e$, at 1 min.

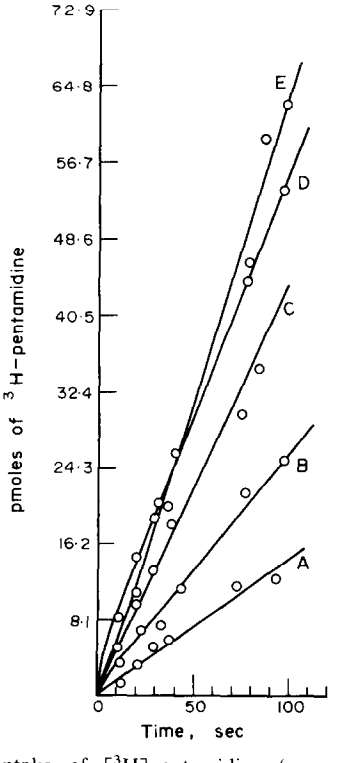

Fig. 3. Uptake of [^{3}H]pentamidine (sp. act., 6·0 $\times 10^{11}$ dis./min/m-mole) by *T. brucei* A (0·34 μM), B (0·64 μM), C (1·30 μM), D (2·60 μM), E (4·50 μM).

of time. This indicates that the system is a saturable function of the external pentamidine concentration. The PCA-soluble layer of a typical uptake experiment during and after the period of time over which the rate is known to be linear was analyzed by t.l.c. (Carrier compound was added to facilitate visualization of pentamidine by ultraviolet.) The radioactivity co-migrated with the unlabeled carrier.

Substrate specificity. To determine the specificity of the pentamidine transport system, other compounds were added to the exposing solution and their effect on initial rates of uptake was studied.

In the presence of the diamidines (Fig. 1), stilbamidine, propamidine and hydroxystilbamidine, the initial rates of pentamidine uptake were reduced. Lineweaver–Burk plots of the data (Fig. 5) are characteristic of competitive inhibition. The K_i values obtained for stilbamidine, propamidine and hydroxystilbamidine were 2·4, 2·2 and 1·7 μM respectively. The addition of benzamidine also resulted in competitive inhibition of pentamidine uptake with a K_i value of 6·5 μM.

It is unlikely that the usual function of this transport system is for the translocation of diamidines; therefore, some metabolically active compounds were tested as possible competitors. The basic amino acids, lysine and arginine, as well as the aromatic amino acids, phenylalanine, tryptophan and histidine, at concentrations as high as 100 times the lowest pentamidine concentration, had no effect on pentamidine uptake. Likewise, PABA and folic acid at 42 times the lowest pentamidine concentration did not inhibit uptake.

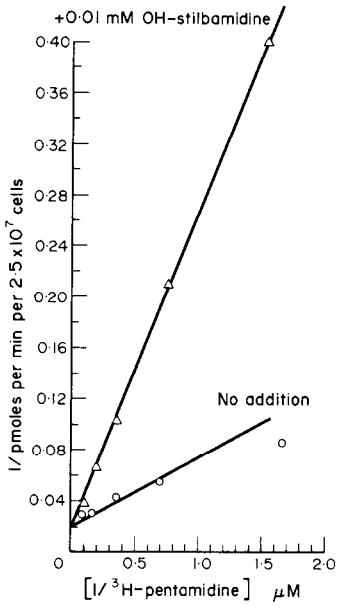

Fig. 5. Double reciprocal plot showing the effect of 0·010 mM hydroxystilbamidine on rates of transfer of pentamidine at various concentrations.

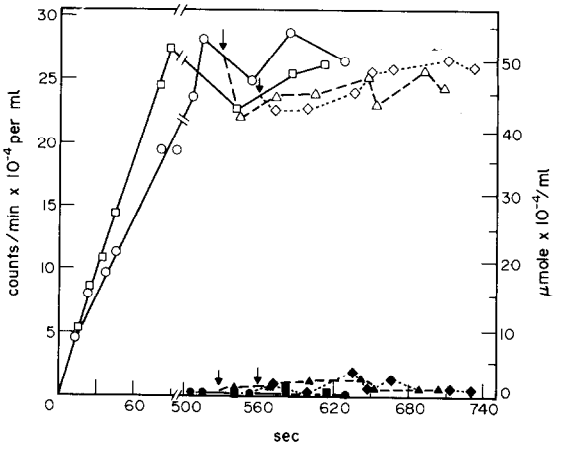

Fig. 6. Exit of [^{3}H]pentamidine from *T. brucei* in the presence and absence of 100 μM pentamidine and hydroxystilbamidine. Points before the break represent pentamidine uptake. Arrows indicate time of addition of 2 ml of the cell suspension to the flask containing external diamidines. Symbols representing intracellular pentamidine: ○, control for pentamidine addition; △, addition of pentamidine; □, control for hydroxystilbamidine addition; and ◇, addition of hydroxystilbamidine. Symbols representing extracellular pentamidine: ●, control for pentamidine addition; ▲, addition of pentamidine; ■, control for hydroxystilbamidine addition; and ◆, addition of hydroxystilbamidine.

Pentamidine efflux. Data presented above show that *T. brucei* accumulates pentamidine against a concentration gradient (Fig. 4B insert). When cells loaded with [^{3}H]pentamidine were removed from the exposing solution by centrifugation and resuspended in HBSSA, there was no loss of radioactivity from the cells nor was there an increase in radioactivity in the external solution.

When pentamidine or hydroxystilbamidine (final concn 100 μM) was added to the external solution, a periodic fluctuation in the intracellular radioactivity and an increase in radioactivity in the external solution occurred (Fig. 6). A maximum of $1{\cdot}5 \times 10^4$ μmoles/ml of external solution excited from 2×10^8 cells within 1 min in the presence of external diamidines.

Effect of metabolic inhibitors on pentamidine uptake. Blood trypomastigote forms of *T. brucei* derive the energy needed to carry out metabolic processes solely from the small amount of ATP generated by glycolysis. This type of respiration uses a great deal of glucose, is cyanide insensitive, and is inhibited by hydroxamic acid derivatives [10]. When SHAM is used to inhibit respiration with glucose as the energy source, the accumulation of pentamidine is inhibited but not competitively (Table 1). This inhibition is concentration dependent, increasing from 3·0 per cent inhibition of control rate at 0·5 mM to 78 per cent inhibition at 5·0 mM. When glycerol is the energy substrate and SHAM is added, pentamidine transport ceases and the parasites die.

Iodoacetate (IAA) is a glycolytic inhibitor, and in the presence of 1·0 mM IAA, pentamidine transport was inhibited 47 per cent. In the presence of both 1·0 mM SHAM and 1·0 mM IAA with glucose as the energy substrate, the trypanosomes became immobile.

Table 1. Effect of metabolic inhibitors on uptake of pentamidine*

Inhibitor	Concentration (mM)	Rate of uptake (pmoles/min/2·5 × 10^7 cells)	% Inhibition
None		21·09	0
SHAM	0·5	20·05	3
SHAM	1·0	9·37	56
SHAM	2·0	8·79	58
SHAM	5·0	4·69	78
IAA	1·0	11·11	47
SHAM + IAA	1·0	8·20	61

* To 0·5 ml of inhibitor was added 0·5 ml of the cell suspension (4×10^8/ml). This suspension was preincubated for 30 sec prior to the addition of 1·0 ml of [^{3}H]pentamidine (2·3 μM). Samples of 250 μl were taken every 10–15 sec, and cells were checked for viability at the end of the experiment.

DISCUSSION

We have determined that net accumulation of pentamidine by blood trypomastigotes of *T. brucei* conforms to Michaelis–Menten kinetics and that other aromatic diamidines competitively inhibit pentamidine uptake. The validity of conclusions drawn and inferences made from the overall results depend upon special safeguards we have taken. The safeguards most relevant to these experiments were: (1) to test the purity of the [^{3}H]pentamidine using physico-chemical and biological parameters; (2) to isolate trypanosomes free from contaminating blood cells and platelets; (3) to use concentrations of pentamidine and competing trypanocidal drugs below concentrations which had any adverse effects on *T. brucei* during 5-min incubations; (4) to monitor and control cell viability before, during and after uptake of trypanocidal drugs; (5) to remove the parasites rapidly and quantitatively from radioactive solutes during uptake studies; (6) to generate double reciprocal plots from initial rates only; and (7) to recover and identify intracellular and extracellular label. Other investigators have reported that pentamidine is not altered by the host's metabolic processes [11,12]. The metabolic fate of pentamidine in trypanosomes has not previously been investigated. The results in the present study using t.l.c. show that pentamidine is not altered by *T. brucei* during initial uptake.

Data from 16 experiments fit the kind of kinetics consistent with a substrate-specific carrier model [13,14]. The K_m value, that concentration at which the carrier sites are half-saturated, consistently falls between 1·5 and 3·1 μM. There is greater variation in the V_{max} value obtained, presumably due to physiological differences from one isolate to another. These differences may be associated with the population density of organisms in the host's blood at the time of isolation, as well as the age of the infection. However, at present we do not have sufficient data to advance such conclusions.

From competition studies, it is apparent that the transport of aromatic diamidines is specific. All of these compounds share a common structure of a straight chain bridge linking two benzene rings with amidino groups in the *para*-position to the bridge. Propamidine, with a K_i of 2·2 μM, is closest to pentamidine in chemical structure. However, it does not compete as well as hydroxystilbamidine, which has a K_i of 1·7 μM. On the other hand, stilbamidine, with a K_i of 2·4 μM, is less effective than propamidine. It differs from hydroxystilbamidine only by the absence of an $-$OH group. Assuming that the K_i values equal K_m values, the K_i values obtained for inhibition of pentamidine uptake by these aromatic diamidines imply a certain order for the affinity of the inhibitor for the transport site; therefore, hydroxystilbamidine should have the highest affinity for the site, followed by propamidine, then stilbamidine. This competitive inhibition may be important in terms of the possible role that this common transport system might play in conferring cross-resistance [15,16] to these trypanocidal agents. It would be speculation to say at this point what specific portion of the molecule is involved in recognition and binding to the transport site. However, the fact that benzamidine inhibits [^{3}H]pentamidine uptake competitively suggests that only one benzene ring with the amidine moiety in the fourth position is required for recognition and that the bridge linking two of these aromatic amidine groups is not involved. Speculating that a pentamidine transport site, shared by other diamidines and benzamidine, is conventionally used for the uptake of some metabolically useful substrate, various metabolically active compounds were added to solutions of pentamidine and their effect on uptake was studied. Neither folic acid nor PABA affected the uptake of pentamidine by *T. brucei*. The lack of inhibition by PABA indicates a requirement for the amidine group on the benzene ring.

Amos and Vollmayer [1] reported that lysine and arginine reversed the inhibitory effect of pentamidine on the growth of *E. coli*. Lysine accumulation and retention were inhibited by pentamidine in *S. aureus* [2] and in *C. fasiculata* [17]. Inhibition of lysine uptake by pentamidine and arginine in *C. fasiculata* prompted Gutteridge [17] to suggest that pentamidine entered this organism via an arginine–lysine transport system. Data presented in this report show no inhibition of pentamidine uptake by these two amino acids and, therefore, do not support the notion that pentamidine shares such a lysine–arginine transport system in *T. brucei*. The inhibition of lysine uptake observed in these other systems by pentamidine may be a reflection of effects pentamidine has on other metabolic processes, such as macromolecular synthesis or energy production. Because of the aromatic nature of pentamidine, the aromatic amino acids were tested for their ability to inhibit pentamidine uptake, again with negative results.

Pentamidine transport in *T. brucei* is concentrative (Fig. 4B insert); this complicates counterflow studies because the pentamidine that moved out of the cells is transported inward again. When *T. brucei* was loaded with [^{3}H]pentamidine, then separated from the exposing solution, and finally resuspended in HBSSA in the absence of external diamidines, [^{3}H]pentamidine was retained (Fig. 6). Intercellular [^{3}H]pentamidine trapped during centrifugation was moved into the cell against a concentration gradient of 200:1. In the presence of 100 μM pentamidine or hydroxystilbamidine, there was minimal stimulation of tritium exit. There was an increase in counts for the first 40–60 sec, after which the number of counts in the external solution decreased. This decrease indicates re-entry of label into the cells and supports the notion that pentamidine transport is concentrative. Trapping of [^{3}H]pentamidine by *T. brucei* 110 M due to intracellular binding to dihydrofolate reductase and nucleic acids may contribute to accumulation of the drug. However, the reductase and nucleic acids do not influence cellular accumulation and concentration of the drug in developmental forms and a drug-resistant strain of *T. brucei* 110. In these organisms pentamidine uptake is markedly reduced or abolished [9].

The strain of *T. brucei* used in these studies, like other virulent laboratory strains, becomes immobile and dies within minutes unless glucose or some other glycolytic substrate is present. It has a poorly developed mitochondrion, lacks a functional tricarboxylic acid cycle and cytochromes and, under aerobic conditions, metabolizes glucose to pyruvate and a trace

of glycerol [7]. Oxidized pyridine nucleotides (NAD) for glycolysis are regenerated from NADH via an α-glycerophosphate oxidase [18]. This system is cyanide insensitive but is inhibited by hydroxamic acid derivatives [8], as is cyanide-insensitive respiration in the yeast-like fungus, *Monilielia tomentosa* [19] and *Neurospora crassa* [20]. Thus, in the presence of SHAM, *T. brucei* must use anaerobic pathways of glycolysis. Ryley [21] has found that blood trypomastigotes of *T. brucei rhodesiense* convert glucose quantitatively to 1 mole pyruvate and 1 mole glycerol under anaerobic conditions, but do not metabolize glycerol anaerobically, presumably because the α-glycerophosphate oxidase system does not convert α-glycerophosphate to dihydroxyacetone phosphate (DHAP). This conversion via the NADH-linked α-glycerophosphate dehydrogenase would result in reduction of pyridine nucleotides and subsequent inhibition of glycolysis. Glucose can, however, be converted to 2 moles triose phosphate, 1 mole being converted to pyruvate and the other to DHAP via the NADH-linked dehydrogenase, resulting in the regeneration of NAD [7].

These metabolic processes are reflected in our transport data. Anaerobic conditions are simulated when SHAM is used to inhibit the α-glycerophosphate oxidase. In the presence of SHAM and IAA, pentamidine transport is inhibited. The inhibition of respiration by SHAM and of glycolysis by IAA results in a reduction of available energy. Since pentamidine transport is also inhibited, a coupling of energy to the pentamidine transport system is inferred.

The data presented in this report show that pentamidine transport in blood trypomastigotes of *T. brucei* is a carrier-mediated, substrate specific, concentrative, energy-coupled process.

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