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Metabolic Effects of a Methylthioadenosine Phosphorylase Substrate Analog on African Trypanosomes

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ABSTRACT. The effects of 5′-deoxy-5′-(hydroxyethylthio)adenosine (HETA), a trypanocidal analog of 5′-deoxy-5′-(methylthio)adenosine (MTA), on polyamine synthesis and *S*-adenosylmethionine (AdoMet) metabolism were examined in bloodstream forms of *Trypanosoma brucei brucei*. HETA was cleaved by trypanosome MTA phosphorylase at the same rate as the natural substrate, MTA, in a phosphate-dependent reaction. Fluorine substitution at the 2-position of the purine ring increased activity by ~50%, whereas substitution with an amino group reduced activity to about one-third of the control. HETA was accumulated by trypanosomes with internal concentrations of 100–250 μM and >800 μM after a 15-min incubation with 1 and 10 μM, respectively. Trypanosomes preincubated with HETA metabolized it at a rate of 21.9 nmol/hr/mg protein. Preincubation of cells with HETA at 1 or 10 μM inhibited spermidine synthesis from [^{3}H]ornithine by 22–37%, and increased the cytosolic levels of AdoMet by 2- to 5-fold and that of MTA by up to 8-fold. *S*-Adenosylhomocysteine (AdoHcy) levels also increased 1.5- to 7-fold in treated cells, whereas decarboxylated AdoMet decreased 65%. Preincubation of trypanosomes with HETA for 4 hr also reduced the incorporation of [^{35}S]methionine in trichloroacetic acid-precipitable material by 50–60%, and reduced the methyl group incorporation into protein from [U-^{14}C]methionine by 65–70%. Thus, HETA interferes with a series of biochemical events involving the participation of AdoMet and methionine in polyamine synthesis, protein synthesis, and transmethylation reactions. BIOCHEM PHARMACOL **57**;1: 89–96, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. polyamine synthesis; protein methylation; *S*-adenosylmethionine metabolism; methionine recycling

Human and veterinary African trypanosomiasis is a prominent health and economic concern in Sub-Saharan Africa [1]. Development of new agents against African trypanosomes has been hindered by the lack of specificity and toxicity, with few new agents moving beyond the *in vitro* stage of screening to laboratory models [1]. In particular, agents targeting polyamine metabolism have demonstrated activity *in vitro*, as well as in model infections and clinically. These agents include DFMO§ [2] and MDL 73811 [3, 4], whose respective targets are ODC and AdoMet decarboxylase. Also targeting this pathway is a related agent, HETA [5, 6], a substrate analog of MTA, which is a byproduct of an aminopropyl group transfer from decarboxylated AdoMet in spermidine and spermine synthesis [7]. In eukaryotes, MTA is phosphorolytically cleaved by a specific MTA phosphorylase into adenine and MTR1P [8]. MTR1P is recycled to methionine in a five-step pathway [9, 10], which is one of two major routes to methionine salvage in eukaryotic and prokaryotic cells [11].

HETA is trypanocidal *in vitro* and curative in model laboratory infections of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* [6, 12, 13]. It is a preferential substrate for trypanosome MTA phosphorylase, as opposed to its mammalian counterpart, and appears to act by releasing a toxic analog of MTR1P [6]. HETA is treated as an adenosine nucleoside by the P_2 adenosine transporter in *T. b. brucei* and as an AdoMet analog by a novel, specific AdoMet transporter recently found in trypanosomes [14, 15]. In this study, we examined the concentration and metabolism of HETA by intact trypanosomes, and its

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§ *Abbreviations:* DFMO, DL-α-difluoromethylornithine; AdoMet, *S*-adenosylmethionine; HETA, 5′-deoxy-5′-(hydroxyethylthio)adenosine; MTA, 5′-deoxy-5′-(methylthio)adenosine; MTR1P, 5-deoxy-5-methylthioribose-1-phosphate; ODC, ornithine decarboxylase; MDL 73811, 5′-{[(Z)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine; TSG, Tris–saline–glucose buffer (0.1 M Tris–HCl, pH 7.4, containing 0.9% NaCl, 40 mM glucose + 50 U penicillin, 50 μg/mL of streptomycin); TCA, trichloroacetic acid; AdoHcy, *S*-adenosylhomocysteine; and PS:G, phosphate–saline–glucose buffer (0.1 M $NaPO_4$ buffered 0.9% NaCl, 1% glucose, pH 7.4, + 50 U penicillin, 50 μg/mL of streptomycin).

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effects on parasite AdoMet and polyamine metabolism, to develop a better understanding of its action on the parasite and to guide the synthesis of related agents.

MATERIALS AND METHODS

Trypanosome Strains

The *T. b. brucei* Lab 110 EATRO isolate and *T. b. rhodesiense* KETRI isolate (2538) were maintained and passaged in rats as described previously [6]. The bloodstream-form trypanosomes used throughout this study were obtained from rats as 3- to 5-day infections and harvested by cardiac puncture after CO_2 anoxia. Trypanosome suspensions were separated from formed blood elements by DEAE cellulose chromatography in TSG buffer, washed in TSG, and resuspended in various media or buffer, depending on their use.

Whole Cell Studies

After harvesting, trypanosomes used for metabolic studies were suspended (5×10^6/mL) in modified Iscoves medium containing 20% fetal bovine serum (HMI-18; [16]) and maintained overnight at 37°. Then cells were resuspended in HMI-18 plus the appropriate label and additions. Pretreatment with HETA or other agents for 1–4 hr was done in this medium minus methionine at 37° in a CO_2 incubator (4% CO_2). After pretreatment, cells were centrifuged (3400 *g*) and resuspended in fresh medium with appropriate agents and radiolabel. [2,3-^{3}H-(N)]Ornithine (40 Ci/mmol) or [^{35}S]methionine (1175 Ci/mmol) was used to examine polyamine synthesis (putrescine, spermidine) and to detect intermediates [AdoHcy, MTA, decarboxylated AdoMet] in methionine/AdoMet metabolism, respectively. The HPLC system used has been described [17, 18].

Incorporation of methionine into protein was measured as described [19, 20] by preincubating cells with 1 or 10 μM HETA for 2 hr, then washing and resuspending cells in HMI-18 minus methionine, but including [^{35}S]methionine (4.0 nmol, 1 mCi) plus HETA for 6 hr, stopping the incubation with 10% TCA, and filtering the precipitate (25 mm GFB filters). The filters were washed twice with 10% TCA, once with 10% TCA + 40 mM methionine, and twice with ethanol to remove lipids. Then the filters were air-dried and counted. Protein methylation was measured in similar incubations as the amount of [U-^{14}C]methionine incorporated into TCA-precipitable cell fractions. TCA precipitates (10%) of treated cells were filtered, washed, dried, and counted [19, 21].

Enzyme Studies

Cell-free extracts were made from washed (TSG) trypanosome suspensions (2×10^8/mL). For AdoMet decarboxylase, cells were frozen–thawed three times in "breakage medium" (44 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.05 mM pyridoxal-5′-PO_4; 60 mM phosphate buffer, pH 7.0), and the cleared (3400 *g*) supernatant was used in a reaction mixture containing 220 μL breakage medium, 1 mM putrescine–2HCl, 0.2 μCi *S*-[*carboxyl*-^{14}C]adenosylmethionine (0.2 mM), and 250 μL (0.5 to 0.6 mg protein) enzyme preparation. Evolution of $^{14}CO_2$ was measured in stoppered vials as described [22].

MTA phosphorylase assays were quantitated in two ways: spectrophotometrically at 305 nm by measuring the production of dihydroxyadenine over 1 hr in a xanthine oxidase-coupled reaction [23], and by measuring adenine formation directly by HPLC assay of 10% TCA-precipitated reaction mixtures at 254 nm (retention time: 23:00 min; [17, 18]).

AdoMet synthetase was measured as described in Yarlett *et al.* [24].

Protein Determination

Proteins were determined by the method of Lowry *et al.* [25] using bovine serum albumin as standard.

Chemicals

The following radiolabeled compounds used were obtained from Dupont, NEN Research Products and included [^{35}S]methionine (1175 Ci/mmol); L-[U-^{14}C]methionine (290 mCi/mmol); and L-[2,3-^{3}H(N)]ornithine (40 Ci/mmol). *S*-[*carboxyl*-^{14}C]Adenosylmethionine (56 mCi/mmol) was obtained from Moravek Biochemicals Inc. HETA and its 2-fluoro and 2-amino analogs were synthesized according to Sufrin *et al.* [5].

RESULTS

HETA Uptake and Utilization

Bloodstream trypanosomes transport adenosine through two sites, P_1 and P_2 [26], while AdoMet is transported through a separate adenosine- and inosine-independent site [14, 15]. HETA at 1 μM interferes significantly with the uptake of both adenosine (P_2 site) and AdoMet in short-term uptake studies (30% inhibition of each; [14, 15]). In this study, we examined internal concentrations of HETA in parasites incubated with 1 or 10 μM HETA for 15 min. Table 1 lists the results of these separate experiments. Using HPLC and UV quantitation (254 nm) of TCA extracts, internal HETA concentrations approached 1 mM with a 10 μM external concentration and 100–250 μM with 1 μM external levels (cell volume: 58 μL/10^9 trypanosomes; [27]). The internal HETA concentrations found in whole cell incubation studies—roughly 100 times the external concentrations—reflect unmetabolized agent only. We estimated HETA utilization in an experiment in which trypanosomes (10^9/mL in PS:G) were preincubated for 10 min with 100 μM HETA, then washed, and suspended in buffer without HETA. At 0, 30 sec, and 1, 5, and 10 min after resuspension, 200-μL aliquots were withdrawn and centrifuged (12,000 *g*) through mineral oil, washed in

TABLE 1. Internal concentration of HETA in bloodstream trypanosomes after incubation *in vitro*

Experiment	External HETA (μM)	Internal concentration (μM)
1	10	860 ± 180
2	10	885 ± 124
3	1	122 ± 20
4	1	258 ± 28

Trypanosomes (10 mL of 5×10^6/mL) were incubated in PS:G buffer. After 15 min, the cells were centrifuged, washed, and lysed with 10% TCA. HETA in supernatant preparations was quantitated using HPLC analyses and UV detection (254 nm). Results are from incubations in triplicate for each experiment (± SD).

PS:G, and lysed with 10% TCA. After overnight storage at 0–4°, the extracts were assayed for HETA by HPLC as described in Materials and Methods. Cells at "0" time post-incubation contained 765 ± 35.5 nmol HETA/mL of cytoplasm (8.875 ± 0.4 nmol/2 × 10^8 cells) and 354 ± 58.6 nmol/mL of cytoplasm (4.11 ± 0.68 nmol/2 × 10^8 cells) after 10 min, a decrease of 411 nmol/mL (4.76 nmol converted/2 × 10^8 cells or 3.66 nmol/mg protein). Utilization of HETA under these conditions corresponds to 2466 nmol/hr/mL of cytoplasm (21.9 nmol/hr/mg of protein or 28.56 nmol/hr/2 × 10^8 cells).

Dialyzed crude trypanosome MTA phosphorylase preparations cleave HETA to adenine and 5-hydroxyethylthioribose-1-phosphate at a rate equal to that of MTA in a reaction that is 43% dependent on exogenous phosphate ([6]; Table 2). Table 2 lists the activity of HETA and several other MTA analogs as substrates for trypanosome MTA phosphorylase, as determined by HPLC analysis. HETA at 200 μM was cleaved at the same rate as MTA in the presence of phosphate. The rate obtained for both substrates without phosphate was 43% lower in each reaction. Addition of a fluoro group on the 2-position of the adenine moiety in HETA yielded a compound (2-fluoro-HETA; Fig. 1) that was cleaved at nearly 1.5 times the rate

TABLE 2. *T. b. brucei* MTA phosphorylase activity with MTA and substrate analogs

Substrate	Purine liberated (nmol/mg protein/hr)	% of MTA + PO_4
MTA + PO_4	10.15	100
MTA − PO_4	5.8	57
HETA + PO_4	10.15	100
HETA − PO_4	5.8	57
2-fluoro-HETA + PO_4	15.08	148
2-fluoro-HETA − PO_4	10.8	106
2-NH_2-HETA + PO_4	2.9	29
2-NH_2-HETA − PO_4	1.85	18

Reaction mixtures contained 0.05 M Tris–HCl (pH 7.4), 200 μM substrate, and 0.4 mg cell extract in a final volume of 250 μL. KPO_4 was added at a final concentration of 1.65 mM. Mixtures were incubated for 60 min at 37° and stopped by the addition of 25 μL of 40% TCA at 0–4°. After overnight incubation, preparations were centrifuged, and the supernatants were analyzed by HPLC and UV quantitation of the purine base liberated (254 nm). All reactions were in duplicate. Standard curves were run with 2-fluoro adenine, 2-NH_2 adenine, and adenine.

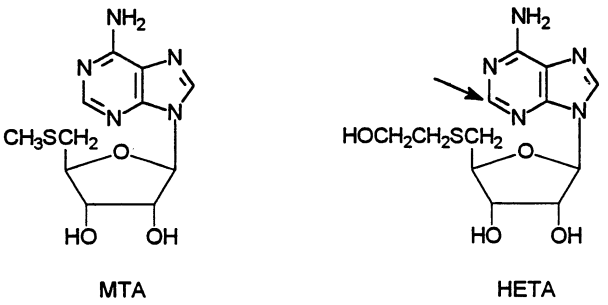

FIG. 1. Structures of 5′-deoxy-(methylthio)adenosine (MTA) and 5′-deoxy-5′-(hydroxyethylthio)adenosine (HETA). The position of the 2-fluoro- and 2-amino-substituents of 2-fluoro-HETA and 2-amino-HETA is designated by an arrow.

of HETA or MTA. This reaction also showed phosphate-dependency, operating at an approximately 30% lower rate in the absence of added phosphate. Substitution of an amino group on the 2-position (2-amino HETA; Fig. 1) resulted in a 71% decrease in activity from rates obtained with MTA and HETA in the presence of phosphate.

Effect on Polyamine Metabolism

A series of experiments was undertaken to determine whether HETA affected polyamine synthesis. In each experiment, bloodstream-form trypanosomes were preincubated with 10 μM HETA for 1 hr, and then were post-incubated with 10 μCi [^{3}H]L-ornithine for 1 hr in the presence of HETA. TCA extracts were then analyzed by HPLC separation. In two experiments, 500 μM unlabeled L-methionine was also included in the post-incubation medium as a source of AdoMet for spermidine synthesis. Table 3 shows the results of these experiments. Putrescine synthesis remained relatively unchanged during exposure to HETA in all five experiments; however, spermidine synthesis decreased 22–37%. In the experiments in which 500 μM methionine was included, spermidine synthesis in untreated controls was 2- to 8-fold above controls without methionine. Nevertheless, HETA caused approximately the same degree of reduction in spermidine content (23–34%) as in the experiments without added methionine.

In a separate study, the fate of [^{35}S]methionine was studied in cells pretreated with HETA for 1 or 2 hr, and then coincubated with HETA + 4 nmol [^{35}S]methionine for an additional 0.5 to 2 hr. Figure 2 gives results of duplicate TCA extracts of treated and control cells that have undergone HPLC analysis [17]. Most noticeable were changes in the levels of AdoMet and MTA. In the cells pretreated with 1 μM HETA for 1 hr, AdoMet was 64% higher than in control cells, and maintained ≥70% elevated levels over the 2-hr incubation (Fig. 2A). MTA levels in 1-hr pretreated cells were 50–150% higher than in controls over the 2-hr incubation. The amount of label incorporated in AdoMet and MTA in 1-hr pretreated cells (1 μM HETA) was 60–90% above that in controls (0.62 to 1.47 pmol/mg protein vs 1.02 to 2.48 for treated). The

TABLE 3. Effect of HETA on polyamine synthesis in *T. b. brucei*

Experiment		Putrescine (pmol/mg protein/hr)	Spermidine (pmol/mg protein/hr)
1	Control	15.6 ± 2.25	0.52 ± 0.07
	+ 10 μM HETA	16.2 ± 2.53 (+4%)	0.33 ± 0.12 (−37%)
2 (+ 500 μM methionine)	Control	6.11 ± 0.17	4.13 ± 0.45
	+ 10 μM HETA	5.12 ± 0.3 (−16%)	2.74 ± 0.25 (−34%)
3	Control	17.96 ± 0.94	0.83 ± 0.23
	+ 10 μM HETA	19.2 ± 6.4 (+7%)	0.65 ± 0.12 (−22%)
4 (+ 500 μM methionine)	Control	2.53 ± 1.16	2.25 ± 0.85
	+ 10 μM HETA	2.35 ± 0.29 (−7%)	1.73 ± 0.08 (−23%)
5	Control	32.7 ± 5.3	1.8 ± 0.45
	+ 10 μM HETA	33.8 ± 2.74 (+3%)	1.16 ± 0.12 (−32%)

Purified and washed bloodstream trypanosomes were preincubated (5×10^6 cells/mL) with 10 μM HETA for 1 hr in PS:G. Then cells were washed and resuspended in PS:G + 10 μM HETA + 10 μCi [^{3}H]L-ornithine. After 1 hr, cells were harvested and washed with PSG, and 250 μL of cold 10% TCA was added to the pellets. TCA extracts were analyzed by HPLC using a flow-through radio detector. Results reflect triplicate determinations in each experiment (± SD). Percentages in parentheses indicate rate of HETA-treated vs control.

pattern of elevated ^{35}S-labeled metabolites was more evident in cells pretreated for 2 hr with 1 μM HETA (Fig. 2B). Cells pretreated for 2 hr with 1 μM HETA had a >5-fold increase in AdoMet after 30 min post-incubation (Fig. 2B) and maintained a >2.7-fold higher level at the 2-hr post-incubation point. Accompanying the elevated AdoMet levels in the treated cells were 5- to 8-fold increases in MTA levels over the 2-hr post-incubation. The total [^{35}S] incorporation of AdoMet + MTA increased to 3- to 5-fold above controls in cells pretreated with 1 μM HETA (Fig. 2B). In addition to the changes in AdoMet and MTA seen in these experiments, a 65% reduction in decarboxylated AdoMet levels was also found in cells pretreated with 1 μM HETA for 2 hr (not shown). In the above experiments, AdoHcy levels in cells pretreated with HETA for 1 hr did not differ from controls; however, cells pretreated for 2 hr had 0.5- to 7-fold increases in AdoHcy values over controls after a 2-hr post-incubation: control values (as pmol/mg protein) 0.5 hr, 0.08; 1 hr, 0.08; 2 hr, 0.098; 1 μM HETA 0.5 hr, 0.55; 1 hr, 0.59; 2 hr, 0.55. These data strongly suggest that HETA, despite its rapid metabolism in trypanosomes, interferes with several aspects of AdoMet function.

Effect on Enzymes of Polyamine Metabolism

HETA was examined for its effects on crude preparations of trypanosome AdoMet synthetase and AdoMet decarboxylase. Concentrations up to 250 μM were not inhibitory to AdoMet synthetase (sp. act.: 9 nmol/mg protein/hr), while concentrations of 250–1000 μM gave an IC_{50} value of 215 μM for AdoMet decarboxylase (sp. act.: 1.27 nmol/mg protein/hr).

Effect on Methylation of Protein

Trypanosomes rapidly incorporate [^{35}S]methionine into AdoMet [17], and utilize [^{14}C-*methyl*]- or [U-^{14}C]methionine for methylation of protein at a rate 8–10 times that of incorporation of [^{35}S]methionine into protein structure [19]. We examined protein methylation in *T. b. brucei* pretreated for 1 hr with 1 μM HETA and then incubated with [U-^{14}C]methionine. Trypanosomes (10 mL of 5×10^6 cells/mL) were then incubated with 1 μM HETA and 85 pmol/mL (0.2 μCi) [U-^{14}C]methionine for up to 6 hr. Aliquots were taken at 1-, 2- and 4-hr intervals, and the filtered 10% TCA precipitates were washed, dried, and counted [19]. Data for triplicate incubations indicated that incorporation of [U-^{14}C]methionine was inhibited 40–46% throughout the incubation period (Table 4). In a separate experiment, cells pretreated for 1 hr with 10 μM HETA had a 68% reduction in [U-^{14}C]methionine incorporation after 6 hr (1510 pmol/mg protein for controls vs 470 pmol/mg protein for HETA-treated cells).

Protein Incorporation of [^{35}S]Methionine

HETA (10 μM) also inhibited [^{35}S]methionine incorporation into TCA-precipitable material. Trypanosomes (*T. b. brucei*) were preincubated with 10 μM HETA for 2 hr, then post-incubated for 6 hr with HETA + 1 mCi (4 nmol) [^{35}S]methionine, precipitated with 10% TCA, and filtered, and the washed precipitates were dried and counted. Incorporation of [^{35}S]methionine was reduced by 64% in *T. b. brucei* (148 pmol/hr/mg protein for the control vs 51 pmol/hr/mg protein for the treated cells). Similar results were obtained with *T. b. rhodesiense* KETRI 2538 in the same experiment: 52% inhibition at 6 hr post-incubation (91.4 vs 44 pmol/hr/mg protein).

DISCUSSION

MTA is produced continuously during polyamine synthesis, as a result of aminopropyl transferase reactions utilizing AdoMet (Fig. 3). In mammalian cells, it is produced via spermidine and spermine aminopropyl transferases, while in African trypanosomes only the spermidine-producing enzyme is active [28]. MTA is rapidly hydrolyzed as it is

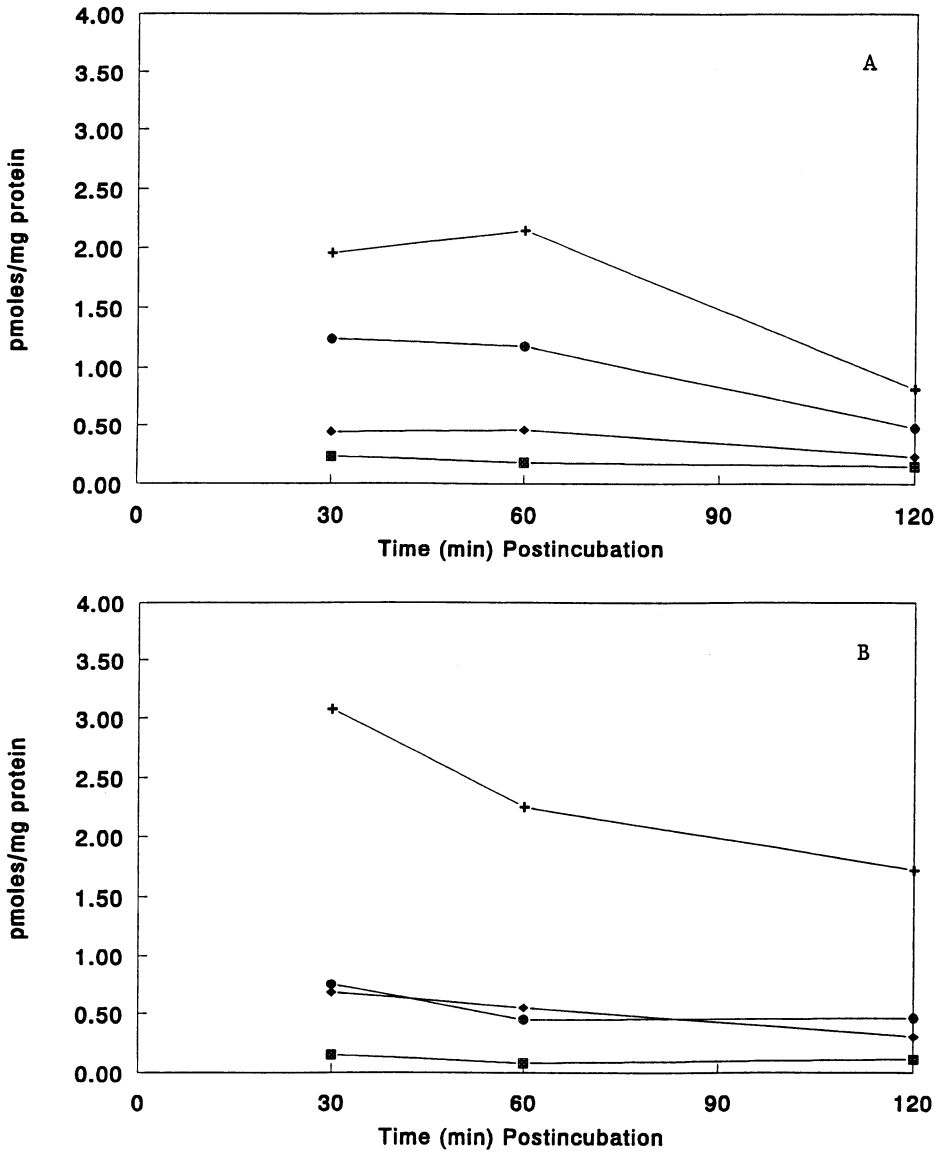

FIG. 2. Effects of HETA on the metabolism of [^{35}S]methionine in *T. b. brucei*. Bloodstream trypanosomes (5×10^6/mL) were resuspended in methionine-free HMI-18 medium and preincubated (37°) for 1 or 2 hr with 1 μM HETA. Then cells were resuspended in fresh medium with HETA + 4 nmol (1 mCi) [^{35}S]methionine (10-mL aliquots). After 30 min, and 1- and 2-hr post-incubation, duplicate flasks were harvested and centrifuged (5000 g for 10 min), and the pellets were washed in TSG. Then the washed pellets were extracted with 250 μL of 10% TCA, and the extracts were assayed for AdoMet and MTA by HPLC. Key: (A) 1-hr pretreatment; (B) 2-hr pretreatment; (●), control AdoMet; (+) HETA AdoMet; (■) control MTA; and (◆) HETA MTA.

formed, an event that reduces its reported effects on the inhibition of mammalian propylamine transferase, AdoHcy hydrolase, and decarboxylation of AdoMet [29–32].

Functionally, in African trypanosomes, MTA phosphorylase may have two critical roles: as a mechanism to salvage adenine for these purine-requiring parasites [33], and as a major means to regenerate methionine in these organisms that heavily methylate protein and lipids (Fig. 3; [19, 20]). A substrate analog of MTA such as HETA, therefore, may impact on three critical areas of trypanosome AdoMet metabolism: the requirement for preformed purines, rapid recycling of methionine for the subsequent formation and utilization of AdoMet in methylation reactions.

Previous work with HETA indicated that it was growth inhibitory *in vitro* at low (10^{-7} M) concentrations [13], and was curative *in vivo* if given in a 7-day treatment regimen vs *T. b. brucei* and *T. b. rhodesiense* isolates in mouse model infections (50–150 mg/kg/day; [6, 12]). The use of osmotic pumps, which deliver 1 μL/hr of HETA (approximately 0.25 μmol/hr at 100 mg/kg) over 7 days, argues for continuous and lengthy exposure for it to be effective against the parasite. Although MTA levels in normal human serum are ≤10 μM [34], the presence of other

TABLE 4. Effect of HETA on [U-^{14}C]methionine incorporated in *T. b. brucei

Time	[U-^{14}C]Methionine incorporation (pmol/mg protein)	
	Control	HETA-treated
1 hr	1042 ± 41.6	614 ± 11.3 (−41%)
2 hr	2303 ± 207.3	1232 ± 66.5 (−46%)
4 hr	4533 ± 87.1	2730 ± 294.5 (−40%)

*Purified bloodstream trypanosomes (10-mL aliquots of 5×10^6 cells/mL) in HMI-18 medium were preincubated for 1 hr with 1 μM HETA, and then were resuspended in HMI-18 minus methionine, but including 2.0 μCi (85 pmol)/mL of [U-^{14}C]methionine. Groups of three flasks were harvested at each time point, washed in HMI-18 minus methionine and HETA, and suspended in 10% TCA overnight. The filters were then washed, dried, and counted [19]. Results are the average of determinations with three batches of cells (± SD).

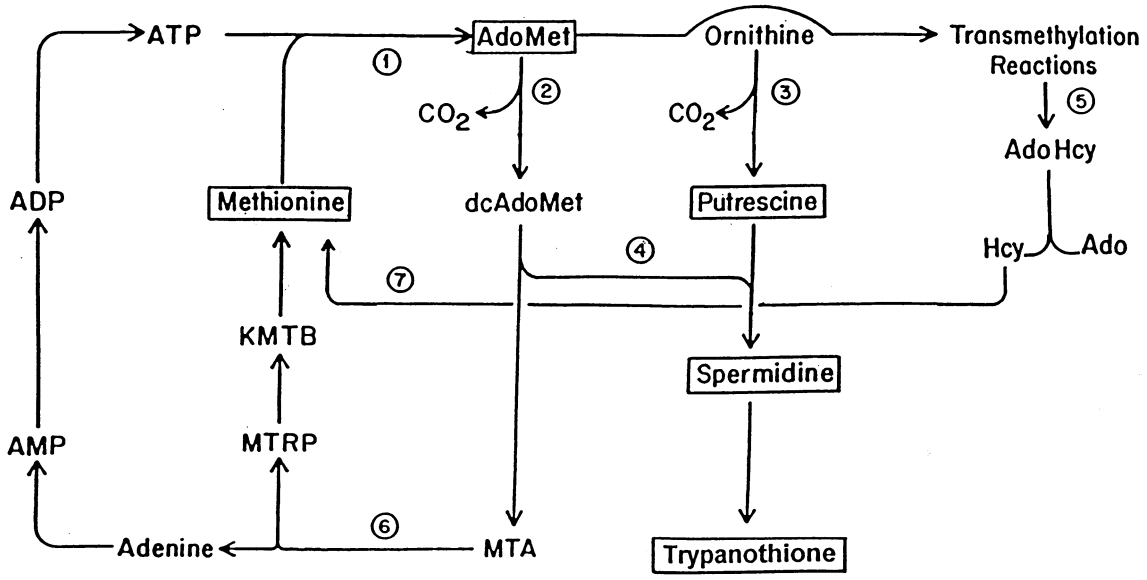

FIG. 3. Metabolism of MTA and AdoMet in trypanosomes. Key enzymes: (1) AdoMet synthetase; (2) AdoMet decarboxylase; (3) ornithine decarboxylase; (4) spermidine synthase; (5) AdoHcy hydrolase; (6) MTA phosphorylase; and (7) 5-methyltetrahydrofolate methyltransferase.

purine nucleosides in the blood might also compete with HETA for uptake and extend the time for effective contact with the agent. In preliminary studies, we found that HETA at 1 μM inhibited uptake of 20 μM [8-^{14}C]MTA by 53% in bloodstream trypanosomes *in vitro* (Goldberg B and Bacchi CJ, unpublished observations).

The present study indicates that HETA is taken up rapidly from the external medium at low concentrations and concentrated by bloodstream-form trypanosomes although the molar values given do not take into account the metabolism of the agent (21.9 nmol/hr/mg protein). Metabolism of HETA by MTA phosphorylase results in formation of hydroxyethylthioribose-1-phosphate, an analog of the natural product methylthioribose-1-phosphate, which is a precursor of methionine in the five-step conversion pathway to methionine [9, 10]. *In vitro*, the growth inhibition due to HETA could be reversed, for the most part, by methionine or ketomethylthiobutyrate, the penultimate intermediate in the recycling pathway [6]. Although this argues for a one-dimensional mode of action (blockade of methionine recycling), the data presented indicate a broader effect of HETA on trypanosome metabolism. HETA interferes with the transport of AdoMet through the specific trypanosome transporter [14, 15], and thus can be regarded as an analog of AdoMet. It is not surprising, therefore, that HETA should exhibit interference with AdoMet-requiring pathways. The observed moderate inhibition of spermidine synthesis (Table 3) suggests that at high concentrations HETA is inhibitory to AdoMet decarboxylase and possibly spermidine synthase. Inhibition of polyamine synthesis was also accompanied by a several-fold elevation of AdoMet, decarboxylated AdoMet, and MTA. It is unlikely that the elevation of AdoMet could be explained by inhibition of polyamine metabolism alone, because large amounts of AdoMet are needed continuously to meet the significant demands for transmethylation reactions in trypanosomes [19, 20]. One means of separating the multiple effects of the intact HETA molecule from that of its methylthioribose analog (hydroxyethylthioribose) would be to compare the effects of HETA to those of an analog that is growth inhibitory *in vitro* but relatively inactive as a substrate analog. This approach is being pursued with several HETA analogs (Sufrin JR and Bacchi CJ, unpublished observations).

In the preincubation studies presented in Fig. 2, one interesting feature was elevation of MTA above controls. In mammalian cells, addition of MTA to growth medium has resulted in inhibition of a number of enzymes utilizing AdoMet, including spermidine and spermine synthases, AdoHcy hydrolase, AdoMet decarboxylase, and transmethylases [29–32]. Although the elevation of MTA in HETA-treated trypanosomes is small in comparison to the elevation of AdoMet, the metabolic effects of elevated MTA in trypanosomes are unknown. One clue may be that in the insect hemoflagellate *Leptomonas spp.* grown in adenine and methionine-free medium, MTA at low (20 μM) concentrations served to partially spare these requirements, but at higher concentrations (>100 μM) it became toxic (Bacchi CJ and Ellenbogen BB, unpublished observations).

In addition to causing elevation of MTA and AdoMet, HETA may also be directly toxic to trypanosomes. As noted, the immediate product of HETA cleavage by MTA phosphorylase is 5-hydroxyethylthioribose-1-phosphate. Meyers and Abeles [35] demonstrated that 5-*S*-ethyl-5-thio-D-ribose was toxic to *Klebsiella* because once phosphorylated by the specific methylthioribose kinase, it was converted through the common five-step salvage pathway

to the cytotoxic methionine analog ethionine. Although it is not clear that the 5-hydroxyethylthioribose-1-phosphate resulting from phosphorolytic cleavage of HETA is carried to hydroxyethionine in trypanosomes, such a possibility leads to interesting questions. For example, trypanosomes have a highly active ketomethylthiobutyrate (KMTB) transaminase with a broad affinity for amino acids [36]; a keto acid analog produced in this pathway might lead to a buildup of KMTB from metabolism of MTA, or to the production of an hydroxyethionine derivative. Although DL-hydroxyethionine itself was not highly inhibitory to *T. b. brucei* growth *in vitro* (19% inhibition at 100 μM: Rattendi D, Sufrin J and Bacchi CJ, unpublished results), this may simply be a consequence of poor uptake by the parasite.

Hydrolysis of MTA (and presumably HETA) by *T. b. brucei* MTA phosphorylase occurs at a rate of 7.5 to 15 nmol/mg protein/hr in the presence of 100–200 μM MTA (Table 2; [23]), while recycling of MTA to methionine was found to occur in undialyzed cell-free extracts at a rate of 2.75 to 5.3 nmol/mg protein/hr (data for *C. fasciculata*; [36]). These rates, coupled with the avid uptake of HETA, indicate that significant levels of hydroxyethylthioribose-1-phosphate and/or its metabolites could be generated by trypanosomes.

HETA is a unique compound in that it affects a cascade of reactions in trypanosomes including polyamine synthesis, AdoMet metabolism, and protein methylation. In this respect, it resembles DFMO, which, although acting on a single target, also causes a cascade of effects. In both cases, these effects seem to be specific for trypanosomes because of critical differences in the biochemical responses of the host cell. HETA is minimally cleaved by mammalian MTA phosphorylase [6], while DFMO does not elevate AdoMet in mammalian cells [37]. HETA cures mouse model infections of *T. b. brucei* and some clinical isolates of *T. b. rhodesiense* [12]. Other *T. b. rhodesiense* isolates transport and cleave HETA but are not eliminated in mouse model infections ([12, 15]; Rattendi D, Goldberg B and Bacchi CJ, unpublished observation). Further studies are needed to determine the mechanism of action of HETA, the reason for constitutive resistance, and the approaches to be taken to broaden its activity.

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